The adipokinetic hormone of the tobacco hornworm, Manduca sexta

Fox, Andrew Mark

Award date:
1989

Awarding institution:
University of Bath

THE ADIPOKINETIC HORMONE OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

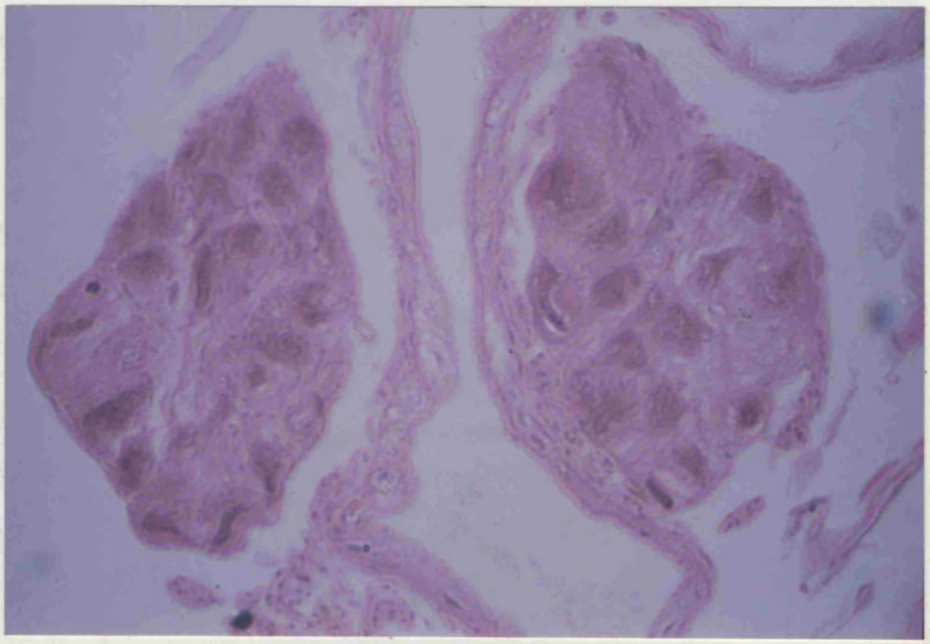
Submitted by Andrew Mark Fox

for the degree of PhD

of the University of Bath

1989

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346



Frontispiece

Transverse section of the corpora cardiaca from an adult *Manduca sexta*, stained with haematoxylin and eosin.

(Magnification x25.6)

In memory of Simon Warden, who prepared the original section.

CONTENTS

ABBREVIATIONS

AMP/cAMP	adenosine 3',5'-monophosphate/cyclic AMP
ATP	adenosine triphosphate
BSOCOES	bis(2-[succinimidooxycarbonyloxy]ethyl)-sulphone
C-terminal	carboxy-terminal
CA	corpora allata
CC	corpora cardiaca
CNS	central nervous system
c.p.m.	counts per minute
Da/kDa	dalton/kilodaltons
DCC1	dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	dicyclohexylurea
Dhbt	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazin
DMAP	dimethylaminopyridine
DMF	dimethylformamide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycolbis(aminoethylether)tetra-acetic acid
FAB-MS	fast atom bombardment mass spectrometry
Fmoc	fluorenylmethoxycarbonyl
HOBT	1-hydroxybenzotriazole
HPLC/RP-HPLC	high performance liquid chromatograph/ reversed phase HPLC
K_m	Michaelis constant
N-terminal	amino terminal

NADP	β-nicotinamide adenine dinucleotide phosphate
NCCI/NCCII	nervi corporis cardiaci I and II
PCMB	parachloromercuribenzoate
Pfp	pentafluorophenyl ester
PTU	phenylthiourea
RIA	radioimmunoassay
RQ	respiratory quotient
SBTI	soybean trypsin inhibitor
tBoc	tertiary butoxycarbonyl
tBu	tertiary butyl
TFA	trifluoroacetic acid
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
u.v.	ultra-violet

Insect Peptides

AKH/AKH-I	locust adipokinetic hormone I
AKH-II(S)/AKH-II(L)	adipokinetic hormone II from *Schistocerca* or *Locusta*
DCC-I/DCC-II	Dipteran corpora cardiaca peptides I and II
G-AKH	*Gryllus* adipokinetic hormone
GPAH	glycogen phosphorylase activating hormone
H-AKH-I/H-AKH-II	*Heliothis* adipokinetic hormones I and II
HTF-II	hypertrehalosaemic factor II
HTH	hypertrehalosaemic hormone
LK-II	leucokinin II
LK-VI	leucokinin VI
M-AKH	*Manduca* adipokinetic hormone

M-I/M-II	myoactive peptides I and II
RoI/RoII	*Romalea* peptides I and II
RPCH	red pigment concentrating hormone

Amino Acids

The standard three-letter abbreviations are used for the amino acid residues in insect peptides. pGlu is the pyroglutamate residue whilst $GlyNH_2$ indicates a C-terminal glycine amide.

LIST OF TABLES AND FIGURES

Page

Page

Page

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Stuart Reynolds for his enthusiasm and guidance throughout this project and my other friends in 6 West for providing a cheerful working environment. I am grateful to Dr. Richard Kinsman of the School of Chemistry for his assistance with the peptide syntheses, to Sue O'Neill for her help with the amino acid analyses and to Dr. Karl Siegert of the University of Aberdeen for his advice on the glycogen phosphorylase assay. Financial support was provided by a University of Bath Research Fund Studentship.

I am grateful to Judy Harbutt for typing this thesis. Finally I am especially grateful to my wife Susie for her love, encouragement and transportation and to God for persevering with me.

ABSTRACT

The lepidopteran adipokinetic hormone M-AKH was synthesized using solid-phase Fmoc chemistry in order to investigate its activity in the tobacco hornworm, Manduca sexta. An M-AKH specific radioimmunoassay was developed and used in conjunction with HPLC to determine the M-AKH content of neural tissues from larval and adult Manduca. M-AKH appears to be located almost exclusively within the corpora cardiaca (CC), with adult moths possessing ten times more peptide (21 pmol/CC) than fifth instar larvae (2.2 pmol/CC). A small amount of immunoreactive material was detected in brain extracts, but HPLC analysis of the extracts suggested that this material is not M-AKH.

Synthetic M-AKH activated fat body glycogen phosphorylase when injected into larvae and mobilized lipid from the fat body when injected into adult moths. A comparison of the dose-response curve for phosphorylase activation by M-AKH with previously published data on the activity of CC extracts suggests that M-AKH is identical to the larval glycogen phosphorylase activating hormone (GPAH), which is released during starvation. This conclusion is supported by the radioimmunoassay of haemolymph from starved and fed larvae which indicated the release of M-AKH during the first hour without food. It was not possible to account for all of the adipokinetic activity of CC extracts in terms of their M-AKH content. The possible presence of an additional adipokinetic factor in the adult CC is discussed.

The pharmacology of the adipokinetic response in adult Manduca was investigated using synthetic AKH/RPCH family peptides. Most of the peptides tested lacked adipokinetic activity at a dose of 50 pmol/insect, however AKH-I from locusts, HTF-II from stick insect and M-II from a cockroach were partial agonists eliciting an increase in haemolymph lipid equivalent to 55-68% of the maximal response to M-AKH. A competition assay in which AKH/RPCH peptides were co-injected with M-AKH indicated that the partial agonists were binding to the same receptor population as the native hormone. The sequence requirements of the Manduca AKH receptor are discussed.

The means whereby M-AKH is inactivated was investigated. A neutral metalloendopeptidase with M-AKH degrading activity was partially purified from larval Manduca haemolymph. The physiological role of this enzyme is unclear but it seems unlikely that it has a primary role in M-AKH inactivation.

CHAPTER 1. METABOLIC REGULATION IN INSECTS

INTRODUCTION

Animal tissues depend upon the maintenance of a supply of metabolic fuel in order to survive and function. Carbohydrates, lipids and proteins may be stored following bouts of feeding and mobilized subsequently in response to an increased demand for metabolic energy. Metabolic regulation is important for all organisms with a varied intake of fuel substances (few animals feed all the time and the nutritional content of each meal probably varies). The demand for energy also varies depending on the level of physical activity or the disease status of the animal, and other physiological conditions such as starvation or reproductive status have a profound effect on the metabolic needs of the animal.

METABOLIC FUELS IN INSECTS

I shall introduce the subject of insect metabolism with particular reference to flight. A more comprehensive review of the biochemistry of insect flight metabolism has been provided by Beenakkers *et al*. (1985c) whilst carbohydrate and lipid metabolism in insects have been reviewed by Friedman (1985) and Downer (1985), respectively.

Insect flight is probably the most metabolically demanding activity known in any animal. The energy requirement of an insect at the onset of flight may be up to one hundred times greater than that of the resting insect (Friedman, 1985). Flightless insects, including larval stages, may also experience large variations in

metabolic demand, particularly during periods of starvation which occur normally during the development of many insects (e.g. Lepidoptera). Both flight and starvation challenge the maintenance of a regulated supply of metabolic fuel to the insect's tissues.

There is no universal metabolic fuel for flight in all insects. The importance of carbohydrate was demonstrated by Jongbloed and Wiersma (1934) who reported that the respiratory quotient (RQ) of flying honeybees (Apis mellifera) was equal to 1, indicating carbohydrate metabolism. Wyatt and Kalf (1957) demonstrated the importance of the disaccharide trehalose when they correlated wing beat frequency with haemolymph trehalose concentration in the blowfly, Phormia regina. In locusts carbohydrate is utilized during the early stages of flight but lipid is the major fuel during sustained flight (Krogh and Weis-Fogh, 1951; Beenakkers, 1973). An early report of the RQ of several lepidopteran species during rest and flight suggested virtually exclusive metabolism of lipids (Zebe, 1953) however it is now known that many Lepidoptera utilize both carbohydrates and lipids (Beenakkers et al., 1985c). Amino acids (in particular proline) are a third form of fuel used in insects. The concentration of proline in flight muscle falls during flight in the tsetse fly, Glossina morsitans indicating its utilization (Bursell, 1963). The role of proline in flight metabolism appears to be very variable between insect species and it will not be discussed further here.

Carbohydrate-utilizing insects possess large stores of glycogen in their flight muscles, haemolymph and in the fat body (the insect equivalent of the mammalian liver and adipose tissue). Flight,

especially its initiation, demands a very high rate of carbohydrate utilization. The glycogen content of locust flight muscle was shown to be reduced by 64% during the first 10s of flight (Rowan and Newsholme, 1979). The open circulatory system of insects results in the slow distribution of fuel substrates, so a high haemolymph concentration of fuel is required during flight. Since trehalose yields twice as much energy per osmotically active particle as glucose, it is more suitable as the primary carbohydrate fuel in insects. Trehalose is synthesised in the fat body following the degradation of stored glycogen (Friedman, 1985).

Lipids are stored in the form of triacylglycerols in the fat body. During flight the lipolysis of triacylglycerols yields monoacylglycerols which are then reacylated to form the specific diacylglycerols that are secreted (Beenakkers et al., 1985a). These diacylglycerols are transported to the flight muscle in association with haemolymph lipoproteins which are recycled following dissociation at the flight muscle (Wheeler and Goldsworthy, 1985a).

In general, predominantly carbohydrate-utilizing insects such as the honeybee are less capable of sustained flight than predominantly lipid-utilizing insects such as the locust. This is probably due to the greater efficiency of lipid metabolism and storage (Beenakkers et al., 1985c).

PEPTIDE HORMONES AND INSECT METABOLISM

Vertebrate-like Peptides

By analogy with vertebrate systems in which insulin and glucagon have major roles in metabolic regulation, insect

metabolism might be expected to be influenced by peptide hormones. A number of studies have reported the presence of insulin-like and glucagon-like substances in insects and have compared their biological activities with those of injected insulin or glucagon (see Kramer, 1985). Insulin-like and glucagon-like substances were identified in the corpora cardiaca-corpora allata (CC-CA) complex of the tobacco hornworm, Manduca sexta by radioimmunoassay (Tager et al., 1976). An insulin-like peptide was subsequently purified from the haemolymph of Manduca larvae and shown to have a similar amino acid composition to vertebrate insulin (Kramer et al., 1982). Insulin-like immunoreactivity was also found in the median neurosecretory cells from the brain of the blowfly, Calliphora vomitoria. This material displayed insulin-like bioactivity when assayed on isolated rat adipocytes or in the blowfly (Duve et al., 1979). The midgut of the locust, Locusta migratoria contains an insulin-like peptide which displays hypoglycaemic activity (Gourdoux et al., 1985). This peptide also stimulates glucose catabolism as does insulin when injected into Locusta (BenKhay et al., 1987).

A hypoglycaemic factor has been reported in the CC of the cockroach, Periplaneta americana which can induce hypolipaemia in the locust but not in the cockroach. Bovine insulin mimicked these effects (Barrett and Loughton, 1987). The cockroach factor may be similar to a hypolipaemic factor isolated from the storage lobe of the locust CC by Orchard and Loughton (1980). Despite this evidence for vertebrate-like peptides with insulin- and glucagon-like bioactivities in a number of insect species the principal hormonal

regulators of insect flight metabolism are peptides which are quite distinct from insulin or glucagon.

Adipokinetic Hormones in Insects

Steele (1961) reported the existence of a hypertrehalosaemic factor in the CC of Periplaneta which could be responsible for the regulation of carbohydrate metabolism in that insect. Some years later two independent reports identified a hyperlipaemic factor in locust CC (Mayer and Candy, 1969; Beenakkers, 1969). It was proposed that this 'adipokinetic hormone' (AKH) controlled lipid metabolism during sustained flight in the locust. Goldsworthy et al. (1972) demonstrated the release of AKH from intrinsic cells within the glandular lobe of the CC and Hekimi and O'Shea (1987) have recently demonstrated the synthesis of AKH within these cells. Locust AKH was sequenced from the CC of two species Locusta migratoria and Schistocerca gregaria by Stone et al. (1976). It was found to be a decapeptide with a similar sequence to that of the red pigment concentrating hormone (RPCH) previously sequenced from the eye stalk glands of two prawn species (Fernlund and Josefsson, 1972). The sequences of these two peptides are:

AKH pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2

RPCH pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH_2

The manner in which AKH regulates locust flight metabolism has been studied extensively (for a recent review see Orchard, 1987). The nature of the humoral influences on AKH release remain unclear,

although they may be related to changes in haemolymph carbohydrate levels. Beenakkers et al. (1985a) reported the suppression of secretomotor cells, which can trigger AKH release, by the resting level of trehalose. They suggested that in the early stages of flight when the haemolymph trehalose level falls, the secretomotor cells become active triggering AKH release. Orchard and Loughton (1981) demonstrated the neural regulation of AKH release by the nervi corporis cardiaci (NCCI and NCCII) which extend to the CC from the brain. Electrical stimulation of NCCII evoked AKH release from the CC whereas stimulation of NCCI did not. However stimulation of NCCI potentiated the release of AKH caused by NCCII stimulation. Octopamine, which is present in the CC and NCCII, is thought to act as a transmitter between NCCII and the CC, causing a change in the intracellular level of cyclic AMP (cAMP) which leads to AKH release (Pannabecker and Orchard, 1986).

The action of AKH on the fat body was thought to be mediated by cAMP. CC extracts have been shown to increase the levels of fat body cAMP, dibutyryl cAMP mimicked CC activity and cAMP levels were shown to be elevated after flight (Gäde and Holwerda, 1976). However Goldsworthy et al. (1986) found that the increase in fat body cAMP following the injection of purified AKH was less pronounced than might have been expected, raising the possibility that the increase in cAMP may not be directly related to lipid mobilization. A recent study using synthetic AKH demonstrated the failure of AKH to increase fat body cAMP at doses that produced a maximal elevation of haemolymph lipid. Actually, AKH appeared to inactivate membrane-associated adenylate cyclase, the enzyme which

catalyses cAMP formation from ATP (Loughton, 1988). Consequently the role of cAMP in mediating the action of AKH on the fat body remains uncertain.

Diacylglycerol released from the fat body in response to the action of AKH, associates reversibly with a haemolymph lipoprotein (known as high density lipophorin, HDLp or Ayellow, Ay) and smaller proteins (known as apoLp-III or C_L-proteins) to form a new lipoprotein complex (known as low density lipophorin, LDLp or A^+) (Wheeler and Goldsworthy, 1985a; Shapiro et al., 1988). This complex is transported to the flight muscle where a membrane-bound lipoprotein lipase hydrolyses the diacylglycerol to free fatty acids which can enter the muscle cell to be metabolised. The activity of the lipase is greatly enhanced when the substrate is the low density lipophorin (A^+) compared with its activity against the resting state lipophorin (Ay), so AKH appears to regulate lipid utilization at the flight muscle indirectly by increasing the level of the preferred substrate (Wheeler and Goldsworthy, 1985b). A direct effect of AKH on lipid utilization has also been proposed. The oxidation of diacylglycerol by flight muscle was shown to be enhanced by AKH and it was suggested that this may be due to the stimulation of carnitine acyl transferase which catalyses the transportation of fatty acyl coenzyme A across the inner mitochondrial membrane to the site of oxidation (Robinson and Goldsworthy, 1977).

The majority of the carbohydrate utilized during the early stages of locust flight can be obtained from the haemolymph pool. However fat body glycogen phosphorylase (which catalyses

carbohydrate mobilization) is activated early on in flight and AKH has been shown to be responsible for this activation (Van Marrewijk et al., 1986). During sustained flight AKH may maintain carbohydrate metabolism at a reduced rate whilst lipid metabolism is stimulated.

AKH has been shown to cause the non-specific inhibition of protein synthesis by locust fat body (Carlisle and Loughton, 1979, 1986). Initially this phenomenon was thought to be an adaptation for maximizing the efficiency of flight metabolism, however this explanation has since been rejected and the true physiological explanation remains unknown (Orchard, 1987).

A number of peptides displaying structural and functional similarities to AKH have been isolated and sequenced from different insect species (Table 1.1). The original locust AKH is now known as AKH-I following the discovery of two additional peptides in Locusta and Schistocerca (Carlsen et al., 1979) AKH-II occurs in smaller quantities than AKH-I and is generally less potent. The physiological role of AKH-II remains uncertain. A number of other insect species have also been shown to possess two AKH/RPCH family peptides. M-I and M-II from Periplaneta are responsible for the hypertrehalosaemic effect originally observed by Steele (1961) and they also display cardioactivity (Scarborough et al., 1984) and myoactivity on a skeletal muscle preparation (O'Shea et al., 1984). The physiological significance of these activities is unknown. HTH appears to be primarily a carbohydrate mobilizing hormone in its cockroach hosts (Gäde and Rinehart, 1986; Hayes et al., 1986) whereas G-AKH is thought to be primarily a lipid mobilizing hormone

in crickets (Gäde and Rinehart, 1987b). The physiological roles of HTF-II, RoI, II, DCC-I, DCC-II and H-AKH-II in their native insects are not known. In the following chapters I shall describe my work on the adipokinetic hormone, M-AKH from the tobacco hornworm, Manduca sexta.

Legend to Table 1.1. Sequences determined by: a) Stone et al., 1976; b) Fernlund and Josefsson, 1972; c) and d) Siegert et al., 1985; e) and f) Witten et al., 1984; g) Ziegler et al., 1985 and Jaffe et al., 1986; h) Gäde and Rinehart, 1987a; i) Gäde and Rinehart, 1986 and Hayes et al., 1986; j) and k) Gäde et al., 1988; k) Gäde and Rinehart, 1987b; l) and m) hypothetical sequences based on Jaffe et al., 1988a; n) Jaffe et al., 1988b.

Table 1.1. The AKH/RPCH family of peptides.

Peptide	Sequence	Source
a) AKH-I	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-$ThrNH_2$	Locusta, Schistocerca
b) RPCH	pGlu-Leu-Asn-Phe-Ser-Pro-Gly-$TrpNH_2$	Pandalus, Leander
c) AKH-II(S)	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-$TrpNH_2$	Schistocerca
d) AKH-II(L)	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-$TrpNH_2$	Locusta
e) M-I	pGlu-Val-Asn-Phe-Ser-Pro-Asn-$TrpNH_2$	Periplaneta
f) M-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-$TrpNH_2$	Periplaneta
g) M-AKH/H-AKH-I	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-$GlyNH_2$	Manduca, Heliothis
h) HTF-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-$ThrNH_2$	Carausius
i) HTH	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-$ThrNH_2$	Nauphoeta, Blaberus
j) RoI	pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-Gly-$ThrNH_2$	Romalea
k) RoII/G-AKH	pGlu-Val-Asn-Phe-Ser-Thr-Gly-$TrpNH_2$	Romalea, Gryllus
l) DCC-I	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-$TrpNH_2$	Tabanidae
m) DCC-II	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-$TyrNH_2$	Tabanidae
n) H-AKH-II	pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-$AsnNH_2$	Heliothis

CHAPTER 2. PREPARATION OF SYNTHETIC MANDUCA AKH

INTRODUCTION

The synthesis of biologically active peptides using solid-phase and solution methods has facilitated the performance of detailed physiological and biochemical studies by providing pure peptide in amounts far greater than could be readily obtained by purification from the native tissue. This is particularly true for the study of insect peptides such as the members of the AKH/RPCH family, where the tissue source is very small and may contain only a few picomoles (pmol.) of peptide. The sequencing of AKH-I required 3,000 corpora cardiaca (CC) pairs from Locusta and Schistocerca (Stone et al., 1976) and despite improvements in the sensitivity of sequencing techniques 300 CC pairs were needed for the sequencing of AKH-II from Schistocerca and Locusta (Gade et al., 1986).

Synthetic AKH-I was prepared soon after the sequence had been elucidated and truncated analogues were synthesized in order to investigate the sequence requirements of the locust AKH receptor (Stone et al., 1978). AKH-I analogues containing modified amino acid residues were synthesized by a combination of solid-phase and solution techniques (Hardy and Sheppard, 1983) and a specifically tritiated analogue of AKH-I has been synthesized and reported to possess full adipokinetic activity in locusts (Muramoto et al., 1984).

An adipokinetic factor was demonstrated in the corpora cardiaca (CC) of the tobacco hawkmoth, Manduca sexta a decade

ago (Beenakkers et al., 1978). This factor elevated haemolymph lipid levels when injected into adult Manduca but not when injected into Locusta. It was suggested that the Manduca adipokinetic factor was different to that isolated from locusts.

Ziegler et al. (1984) isolated the Manduca adipokinetic hormone from acetic acid extracts of adult Manduca CC by reversed-phase HPLC (RP-HPLC). Amino acid analysis revealed a nonapeptide sharing seven amino acid residues with locust AKH-I. The sequence of Manduca adipokinetic hormone (M-AKH) was determined by Ziegler et al. (1985) using fast atom bombardment tandem mass spectrometry. M-AKH is an uncharged peptide with an amino-terminal blocked by a pyroglutamate residue and a carboxy terminal blocked by glycine amide. The sequence of M-AKH is:

pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-GlyNH_2

In order to investigate the biological activities of this hormone in Manduca I have synthesized the native peptide and two analogues [Tyr^1]M-AKH and [$IodoPhe^4$]M-AKH, using the fluorenyl-methoxycarbonyl (Fmoc) chemistry developed recently to permit solid-phase peptide synthesis on a polyamide resin (Dryland and Sheppard, 1986). The synthesis, purification, structural confirmation and bioassay of M-AKH and the two analogues will be described.

MATERIALS AND METHODS

The Pepsynthesiser System

The syntheses were performed by a Pepsynthesiser II (Cambridge Research Biochemicals (CRB), Cambridge, U.K.) in the School of

Chemistry, University of Bath. Fmoc amino acid derivatives were obtained from C.R.B. The Fmoc group protects the amino terminal (N-terminal) of the derivative during the coupling procedure. The carboxy terminal (C-terminal), which couples to the resin or the previously coupled residue, is activated in the form of a pentafluorophenyl ester (Pfp) which reacts more readily than the Fmoc free acid. The Pfp esters of threonine and serine exist as pastes rather than crystalline solids so 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazin (Dhbt) esters of these amino acids were used instead. L-Pyroglutamic acid pentachlorophenyl ester (from Sigma) was used for the coupling of the N-terminal pyroglutamate residue. Those residues which possessed a labile side chain (Ser, Thr, Tyr) were protected by a tertiary butyl ester (tBu) during the assembly. A comprehensive description of the use of Fmoc derivatives in peptide synthesis is provided by Atherton and Sheppard (1987).

The Pepsyn KB resin upon which the peptides were assembled consists of a polyamide gel held in the pores of an inert Kieselguhr matrix. The resin is derivatised with a linking agent (4-hydroxy-methylbenzoic acid) to facilitate the synthesis of peptides which are blocked by an amide or hydrazide group at the C-terminal. The resin loading specified by the manufacturer defines the quantity of peptide which can be synthesized on a given weight of resin and is expressed in milliequivalents of peptide per gramme of resin, where 1 meq equals 1 mmol of peptide. The resin loading for the batch of Pepsyn KB used here was 0.12 meq/g, therefore in order to synthesize 100 mg (0.1 meq) of crude M-AKH, 0.83 g

of resin was required (since 0.1 meq ÷ 0.12 meq/g = 0.83 g).

All the solvents used during the syntheses were obtained from the Aldrich Chemical Co., Gillingham, U.K. and the dimethylformamide (DMF) was redistilled prior to use. A four-fold excess of reagent was used throughout the assemblies which were monitored by the u.v. absorbance of the circulating solvent at 314 nm using a Cecil CE1220 spectrophotometer. The procedure used to synthesize 100 mg of crude M-AKH (theoretical yield) will be described. The analogues were synthesized by the same method, using two reaction columns connected in series. These columns were disconnected from each other for the coupling of the residues which are distinctive to each peptide. The Pepsynthesiser II system is illustrated diagrammatically in Fig. 2.1.

Preparation and Coupling of the First Derivative

The peptides were assembled from the C-terminal with the initial residue being coupled to the resin using an Fmoc symmetical anhydride of the amino acid. The anhydride was prepared freshly from 0.8 meq of the Fmoc free acid which when mixed with dicyclohexylcarbodiimide (DCCI) yielded 0.4 meq of the anhydride. For the synthesis of M-AKH, 238 mg of FmocGlyOH was dissolved in a mimimal volume of dichloromethane (DCM) with the addition of a few drops of dimethylformamide (DMF) in a round bottomed flask. The solution was stirred while 76 mg (0.38 meq) of DCCI, also dissolved in a minimal volume of DCM, was added. After 10-15 min of stirring the symmetrical anhydride had precipitated together with dicyclohexylurea (DCU). Rotary evaporation was used to remove the DCM and the anhydride was

redissolved in DMF. The DCU remained insoluble in DMF and was retained by the sinter when the anhydride was added to the reaction column.

The coupling of the anhydride to the resin was catalysed by 12 mg of 4-dimethylaminopyridine (DMAP), dissolved in a minimal volume of DMF, which was loaded into the reaction column immediately before the FmocGly anhydride. The reaction mixture was recirculated through the column for 2 h to ensure complete coverage of the resin with the first residue. The extent to which the resin had been covered by the first residue was assessed by the qualitative Kaiser test (see below). The initial coupling for the synthesis of the analogues was assessed by the quantitative Kaiser test and by amino acid analysis. During the synthesis of [IodoPhe^4]M-AKH the FmocIodoPhe anhydride was prepared from the FmocIodoPhe free acid (a gift from CRB) and coupled in the manner described for the Gly anhydride.

Fmoc Deprotection and Subsequent Couplings

Once the glycine residue had been coupled to the resin the Fmoc group was removed from the N-terminal to permit the coupling of the next residue. This deprotection step was achieved by a 10 min recirculation of 20% piperidine in DMF through the reaction column. The system was then washed through with DMF to remove all the piperidine prior to the second coupling. For the coupling, 59 mg of the catalyst 1-hydroxybenzotriazole (HOBT) was added to the sample tube followed by 238 mg (0.4 meq) of FmocTrpOPfp dissolved in a minimal volume of DMF. The reaction mixture was pumped through the column until the u.v. absorbance of the eluate (at 314 nm) began to rise, at which point the flow was switched

to the recirculate mode for 40 min. A few granules of resin were then removed for the qualitative Kaiser test and recirculation was resumed while the test was performed. When the test indicated complete coverage by the second residue, Fmoc deprotection was repeated and the next residue was coupled. If the Kaiser test indicated incomplete coupling a second Kaiser test was performed which always indicated completion of the reaction.

Qualitative and Quantitative Kaiser Tests

The completion of each coupling step was assessed by the qualitative Kaiser test (Kaiser et al., 1970). For the synthesis of the analogues, the initial anhydride coupling was assessed quantitatively by the modified Kaiser test (Sarin et al., 1981).

For the qualitative test a few granules of resin were removed from the reaction column, placed in a small, sintered, glass column and washed sequentially in DMF, DCM, diethyl ether, dried under nitrogen and placed in a small pyrex test-tube. One drop of each of the following solutions was added to the granules:-

Kaiser 1. Ninhydrin (500 mg) in ethanol (10 ml)

Kaiser 2. Phenol (40 g) in ethanol (10 ml)

Kaiser 3. 10 mM KCN (2 ml) in pyridine (98 ml)

The tube was heated in an oven at 100°C for 5 min and the colouration of the granules was then inspected. A blue colouration indicated incomplete coupling, white or straw-coloured granules indicated complete coupling.

For the quantitative test a small sample of resin was removed from the reaction column and placed in a sintered glass tube. The resin was deprotected by 4 column volumes of 20% piperidine in DMF and dried by washes with DMF, DCM, ether and finally under nitrogen. Approximately 6 mg of dried resin was removed, weighed and transferred to a small pyrex vial. The reagents used were a combination of the Kaiser solutions such that Reagent A consisted of 10 ml of Kaiser 2 mixed with 100 ml of Kaiser 3, while Reagent B was the same as Kaiser 1. 100 µl of Reagent A and 25 µl of Reagent B were added to the vial containing the granules and to an empty vial. The vials were heated at 100°C in an oven for 10 min and then cooled in cold water. The colour was washed from the resin with 60% ethanol and the washings were pooled and made up to 5 ml in a graduated flask. The absorbance of this solution was measured at 570 nm using a Cecil 272 spectrophotometer. The number of amino groups attached to the resin was calculated as follows:-

1. Concentration of Amine $= \frac{Abs_{570}}{15,000}$ $\frac{Units}{M}$

2. Number of moles of amine $= \frac{Concentration \times 5.0}{1,000}$ mol

3. Number of amino groups on the resin $= \frac{No.\ of\ moles \times 1,000}{Weight\ of\ Resin}$ meq/g

4. Percentage Loading $= \frac{No.\ of\ amino\ groups}{0.12} \times 100$ %

Completion of Assembly and Deprotection

N-terminal deprotection was required for the final residue of [Tyr^1]M-AKH but not for the other peptides which were terminated by a pyroglutamate residue which lacked an Fmoc group.

The completed peptide, still attached to the resin was washed with 25 ml of tertiary amyl alcohol (t-amyl alcohol); 25 ml of glacial acetic acid; 25 ml of t-amyl alcohol; 50 ml of diethyl ether; dried under nitrogen and stored in a weighing vial at -30°C.

The resin was reequilibrated to room temperature prior to the removal of the side chain protecting groups by 95% trifluoroacetic acid (TFA) in water. The resin was mixed with the acid in a sintered tube by bubbling with nitrogen to ensure constant mixing for 20 min. The acid was changed twice and mixing continued for a further 20 min each time. The deprotected peptide was then washed with t-amyl alcohol; DMF; 10% ethyldiisopropylamine in DMF; DMF; t-amyl alcohol; DMF and ether (30 ml of each solvent was pumped through the resin at a flow rate of 2 ml/min). The resin was then dried under nitrogen and desiccated overnight over phosphorus pentoxide at room temperature.

Cleavage of the Peptide from the Resin

30 ml of methanolic ammonia was prepared by bubbling ammonia through aluminium oxide-dried methanol on ice for 1 h. The resin was placed in a round bottomed flask and swollen with a few drops of DMF. The methanolic ammonia was added to the resin and the flask was immediately sealed with a septum cap pierced with a syringe needle attached to a balloon to allow for changes in pressure within the flask. The mixture was left at room temperature for 4 h with occasional swirling of the flask to ensure maximum exposure of the resin to the cleavage solution. Using a Pasteur pipette the resin was then removed, washed with dried methanol

and the washings pooled with the methanolic ammonia containing the cleaved peptide. The crude peptide solution was dried down on a rotary evaporator, resuspended in 10% acetonitrile and loaded onto a primed C_{18} SepPak cartridge (Waters Associates, Milford, U.S.A.). The material eluted from the SepPak between 10% and 60% acetonitrile was lyophilised and resuspended in approximately 500 µl of 10% acetonitrile ready for HPLC purification. Meanwhile the cleaved resin was washed with 20 ml of t-amyl alcohol; glacial acetic acid; t-amyl alcohol; DMF; ether; dried under nitrogen and stored at -30°C.

Purification of the Synthetic Peptides

A Gilson HPLC system was used for the purification of the synthetic peptides. The system consisted of two Model 302 pumps, a Model 702 gradient manager, a Model 802 manometric module, a Model HM Holochrome u.v. detector (all from Gilson International, Villiers-le-Bel, France), a Rheodyne 7125 injection valve and a Rikadenki chart recorder. The gradient manager was operated through an Apple II microcomputer.

The crude peptide was purified by RP-HPLC on a semi-preparative Spherisorb 5 µm C_{18} column (30 cm x 0.8 cm, HPLC Technology Ltd., Macclesfield, U.K.). Solvent A was 0.1% TFA in water, solvent B was 0.1% TFA in acetonitrile (TFA was redistilled in the School of Chemistry, University of Bath; acetonitrile was obtained from Rathburns, Walkerburn, U.K.). The flow rate used was 2 ml/min and the gradient conditions were: 15% B (0-5 min); 15-40% B (5-15 min); 40-60% B (15-35 min); 60-15% B (35-40 min), followed

by a 15 min reequilibration period. The eluate was monitored at 210 nm for small samples or 254 nm for larger samples. The major peak was collected manually and lyophilised in a Speed Vac concentrator (Savant Instruments, Hicksville, U.S.A.).

Analysis of the Purified Peptides

The purified peptides were analysed by RP-HPLC, fast atom bombardment mass spectrometry (FAB-MS) and amino acid analysis. The mass spectrometry was performed by Shell Research Ltd., Sittingbourne, U.K., by courtesy of Dr. P. Jewess on a Finnigan MAT-90 mass spectrometer. The sample was loaded in a thioglycerol matrix and bombarded with xenon gas at an energy of 8 K.ev.

HPLC analyses were performed on a Spherisorb 5 μm C_{18} analytical column (0.46 x 25 cm, HPLC Technology). The flow rate used was 1 ml/min and the eluate was monitored at 210 nm. For the analysis of M-AKH and [Tyr^1]M-AKH the solvents were the same as those used for the purification, however for the analysis of [$IodoPhe^4$] M-AKH solvent A was 0.05 M NaH_2PO_4 adjusted to pH 3.3 with concentrated phosphoric acid and solvent B was 0.05 M NaH_2PO_4 in 60% acetonitrile, adjusted to pH 3.3 as above. Several gradients were used for the analyses. The gradient for M-AKH analysis was 10% B (0-5 min); 10-60% B (5-30 min); 60% B (30-35 min); 60-10% B (35-40 min). For [Tyr^1]M-AKH the gradient was 15-40% B (0-20 min); 40-60% B (20-30 min); 60% B (30-35 min); 60-15% (35-40 min), and for [Iodo-Phe^4]M-AKH the gradient was 20-100% B (0-30 min); 100% B (30-33 min); 100-20% (33-33.1 min).

For the amino acid analysis 10-20 nmol of peptide was lyophilised in an acid-washed pyrex test tube and then dissolved in 6 N HCl (Aristar grade). The tube was drawn out over a flame to produce a narrow neck and placed in dry ice/ethanol to freeze the sample. The tube was removed from the dry ice/ethanol and evacuated on an oil pump as the sample thawed. When the evacuation was completed the tube was sealed over a flame and incubated at 105°C for 24 h to hydrolyse the peptide into its constituent amino acids. The top of the vial was then removed and the hydrolysate dried down overnight in a vacuum desiccator. The sample was resuspended in 200 µl of 25 mM HCl and injected into a Hilger Chromaspek II amino acid analyser. The eluate from the analyser was monitored at 570 nm. For the analysis of the resin during the synthesis of the analogues, a weighed sample of resin was placed in an acid-washed tube and hydrolysed as described above.

Bioassay of the Peptides

The adipokinetic activity of the purified peptides was determined in adult Manduca. A 10 µl sample of peptide at a known concentration was injected into the moth immediately after the removal of a 5 µl sample of haemolymph. A second sample of haemolymph was obtained after 100 min and the change in the level of total haemolymph lipids was determined by a sulphophospho-vanillin method modified from Goldsworthy et al. (1972) and described in Chapter 4.

Hydrogenation of [IodoPhe^4] M-AKH

The [IodoPhe^4] analogue of M-AKH was synthesised with the intention of producing a specifically tritiated analogue of M-AKH [^{3}H-Phe^4] M-AKH by catalytic reduction with tritium gas. A number of preliminary hydrogenation experiments were performed in order to optimise the requirements for the catalytic reduction of [IodoPhe^4] M-AKH. The most successful of these experiments incorporated the following procedure. 5 mg of 10% palladium on calcium carbonate catalyst (British Drug Houses (BDH), Poole, U.K.) was washed in DMF. The catalyst was separated by centrifugation, resuspended in 1 ml of fresh DMF and added to 2 ml of DMF containing 8 µg of [IodoPhe^4] M-AKH in a round bottomed flask. The flask was perfused with hydrogen for 10 min and then stirred for a further 2 h under an atmosphere of hydrogen. The mixture was then placed in a polypropylene centrifuge tube and spun at 2,000 r.p.m. in an MSE Centaur S bench centrifuge. The supernatant was removed and the pellet washed in DMF. The pooled supernatant and washings were lyophilised, resuspended in 10 ml of 20% methanol and loaded onto a primed C_{18} SepPak cartridge. The fraction eluting between 20% and 60% methanol was collected, lyophilised and resuspended in 200 µl of 20% acetonitrile for HPLC analysis.

The success of the hydrogenation was assessed by the same HPLC method described above for the analysis of purified [IodoPhe^4]M-AKH. The eluate was monitored at 210 nm.

RESULTS

A sample of the crude product of the M-AKH synthesis separated

by RP-HPLC, is shown in Fig. 2.2a. The major peak was collected and analysed in order to confirm its identity with M-AKH. Analytical HPLC of the material from the collected peak revealed a single peak (Fig. 2.2b) which coeluted with a sample of synthetic M-AKH supplied by Dr. R. Keller, University of Bonn, F.R.G. (data not shown). The single peak indicated a successful purification, however coelution with a peptide of known sequence is not sufficient evidence to confirm the identity of the purified peptide, so further characterisation was undertaken.

The FAB-MS spectrum of the purified synthetic peptide is shown in Fig. 2.3a which illustrates a scan of the mass to charge ratios (m/z) in the range 1000-1200 Daltons. The principal peak at m/z 1008.6 is consistent with the molecular ion $[M+H]^+$ for M-AKH which has a mass of 1008 Daltons. The peak at m/z 1172.2 is the $[Cs_5I_4]^+$ internal standard. The peak at m/z 1030.5 is interpreted as the $[M+Na]^+$ ion.

The peptide therefore has the appropriate HPLC characteristics and molecular mass for M-AKH but these results do not reveal any details about the amino acid composition of the peptide. Fig. 2.3b shows the amino acid analysis of the synthetic peptide. Tryptophan is labile under the hydrolysis conditions used so that its presence is inferred by comparison with the HPLC and FAB-MS data. The peptide consists of 2 Thr, 2 Ser, 1 Glu, 1 Gly, 1 Leu and 1 Phe residue which, apart from the absence of Trp is consistent with the peptide being M-AKH.

The quantitative Kaiser test was performed on samples of resin coupled with the Gly anhydride for the synthesis of the M-AKH

analogues. The results indicated a very low coverage of the resin (25%) and repetition of the coupling produced no significant increase in the apparent percentage coverage. Weighed samples of the resin were then deprotected and amino acid analysed (Table 2.1). This analysis, contrary to the Kaiser test, indicated complete coverage of the resin with an absolute loading of 0.13 meq/g for glycine and 0.12 meq/g for the resin standard norleucine consistent with the loading of 0.12 meq/g specified by the manufacturer. It was assumed that the amino acid analysis was more reliable than the Kaiser test and the synthesis was continued.

The purification of [Tyr^1]M-AKH is illustrated in Fig. 2.4. The purified peptide eluted 3.5 min after M-AKH indicating a different chemical identity for this peptide. The FAB-MS spectrum for this peptide (Fig. 2.5a) shows a molecular ion at m/z 1060.6 which is consistent with the $[M+H]^+$ ion for [Tyr^1]M-AKH. The peak at m/z 1082.7 is probably due to the $[M+Na]^+$ ion whilst the small peak at m/z 1044.6 is consistent with the acylium ion formed by the loss of NH_2 from the C-terminal amide. Amino acid analysis revealed the following amino acid composition for the peptide: 2 Thr, 2 Ser, 1 Gly, 1 Leu, 1 Tyr, 1 Phe. The norleucine peak is due to the presence of this amino acid as an internal standard during this analysis (Fig. 2.5b). In combination these data confirm the identity of the peptide as [Tyr^1]M-AKH.

The synthetic [IodoPhe^4]M-AKH also eluted later than M-AKH when analysed by HPLC (Fig. 2.6b). FAB-MS of this peptide revealed a major peak at m/z 1134.5 consistent with the molecular ion $[M+H]^+$ for [IodoPhe^4]M-AKH. The peak at m/z 1118.5 is interpreted as the

acylium ion for this peptide while the peak at m/z 1156.5 is the $[M+Na]^+$ ion (Fig. 2.7a). Amino acid analysis revealed the absence of a phenylalanine residue and the peak eluting slightly later than the trace of histidine is interpreted as the IodoPhe residue (Fig. 2.7b). The amino acid composition data are therefore consistent with the identification of this peptide as [IodoPhe^4] M-AKH.

The adipokinetic activity of the three peptides was assayed in adult Manduca (Table 2.2). M-AKH caused an increase of 33 mg/ml at both of the test doses and it was therefore assumed that this represented the maximum response. [Tyr^1] M-AKH did not produce a significant change in the level of haemolymph lipid when compared with the control. [IodoPhe^4]M-AKH elicited an increase equivalent to approximately 50% of the maximum response, at a dose of 20 pmol however the response to 5 pmol was not significantly different to that of control insects. It would appear that the N-terminal pyroglutamate residue is vital for adipokinetic activity whereas the replacement of a hydrogen atom with an iodine atom at the Phe^4 position reduces but does not remove all the adipokinetic activity.

The results from the hydrogenation experiments varied greatly. The most successful experiment is illustrated in Fig. 2.8. In a control flask the [IodoPhe^4] analogue was mixed with washed catalyst but not exposed to hydrogen. The peptide was less easily recovered than from the experimental flask and the peptide peak corresponded with the unreacted analogue (Fig. 2.8a). In the experimental sample the broad peak shown in Fig. 2.8b is believed to be due to contamination from the DMF. The peptide peak coeluted with M-AKH approximately 2 min earlier than the [IodoPhe^4]

analogue. HPLC analysis suggests virtually complete hydrogenation of the peptide and a yield of approximately 40%. Unfortunately this result was not reproducible and the use of dithiothreitol to poison the catalyst also failed to improve the procedure.

DISCUSSION

Locust adipokinetic hormone (AKH-I) has been synthesised by a variety of techniques since it was sequenced in 1976. Broomfield and Hardy (1977) synthesised the N-terminal hexapeptide by a solid phase method using tertiary butoxycarbonyl (tBoc) amino acid derivatives (see Merrifield, 1986) and coupled this fragment to the C-terminal tetrapeptide which had been synthesised in solution by stepwise addition of amino acid residues to threonine amide. Yamashiro *et al.* (1981) synthesised AKH-I using solid-phase chemistry only whereas Kisfaludy *et al.* (1986) synthesised AKH-I using a pentafluorophenyl ester solution method. The latter group protected the threonine residues in the assembled peptide with benzyl groups. Unfortunately they were unable to remove all of these by catalytic hydrogenation without observing partial saturation of the indole ring of the [Trp^8] residue. Consequently [$Thr(Bzl)^5$]AKH-I was a major by-product of this synthesis.

Analogues of AKH-I were synthesised and produced by enzymatic cleavage of synthetic AKH-I by Stone *et al.* (1978) for use in the investigation of structure-activity relationships. Hardy and Sheppard (1983) synthesised two analogues of AKH-I containing modified amino acid residues by solid-phase and solution methods for use as precursors of a tritiated analogue of AKH-I. Recently,

Moshitzky et al. (1987) synthesised [Glu^1] AKH-I by a tBoc solid phase method for use in the preparation of a radioiodinated analogue of AKH-I.

Solid-phase peptide synthesis using Fmoc chemistry has a number of advantages over solution synthesis or tBoc methods. Solid-phase synthesis by Fmoc or tBoc chemistry is likely to produce a higher yield of peptide than solution synthesis because the peptide is secured on the resin and therefore losses during the assembly due to the transfer of solutions between flasks are greatly reduced. Excess reagents are easily separated from the peptide using a continuous-flow system as described by Dryland and Sheppard (1986). Solid-phase synthesis using tBoc chemistry requires the use of corrosive reagents such as hydrogen fluoride which is commonly used for side-chain deprotection and cleavage of the peptide from the resin on completion of the assembly (Merrifield, 1986). Fmoc solid-phase synthesis is accomplished with milder reagents. The Fmoc group is removed by 20% piperidine in DMF, whereas tBoc groups are removed by 50% trifluoroacetic acid (TFA) in DCM. Side-chain deprotection and peptide cleavage from the resin, as reported here, were achieved using 95% TFA in water and methanolic ammonia, respectively. The separation of the deprotection and cleavage steps reduced the risk of unwanted reactions between the exposed side chains and components of the reaction mixture as the latter solution was circulated through the resin rather than mixed with it in a flask. Atherton et al. (1983) synthesised β-endorphin by the tBoc and Fmoc methods and observed a fourfold increase in yield and enhanced purity using the Fmoc system.

The insect peptide M-AKH and two nonapeptide analogues were successfully synthesised using a solid-phase Fmoc method. The assembly was readily monitored by u.v. absorbance of the circulating solvent and the qualitative Kaiser test for the completion of each coupling step was easily performed and caused minimal disruption of the synthetic process. Unfortunately the quantitative Kaiser test did not appear to be reliable in this instance. It has been successfully applied in the assessment of coupling to other Pepsyn resins (R.G. Kinsman, personal communication), but the result obtained for the coupling of glycine anhydride to the Pepsyn KB resin was not in agreement with amino acid analysis data. The final yield and purity of the crude peptide analogues suggested that the low percentage coverage indicated by the Kaiser test was not correct. The reason for the failure of the quantitative Kaiser test is not known.

The structural identities of the three peptides was confirmed by RP-HPLC, amino acid analysis and FAB-MS. A preliminary bioassay of synthetic M-AKH for adipokinetic activity indicated bioactivity corresponding with that of natural M-AKH (R. Keller, personal communication). Both of the analogues were inactive at doses below 5 pmol and the [Tyr^1] analogue was also inactive at a dose of 20 pmol. These results will be further discussed in a subsequent chapter on the pharmacology of the M-AKH receptor.

[$IodoPhe^4$]M-AKH was synthesised to act as a substrate for the preparation of a specifically tritiated analogue of M-AKH. Unfortunately I was unable to optimise the conditions for the catalytic reduction of the analogue. Consequently a tritiated

analogue of M-AKH has yet to be produced. [Tyr^1] M-AKH was synthesised to act as a substrate for the production of an M-AKH antigen following conjugation to thyroglobulin. The production of such an antigen and the development of a radioimmunoassay using radioiodinated [Tyr^1]M-AKH will be described in the next chapter.

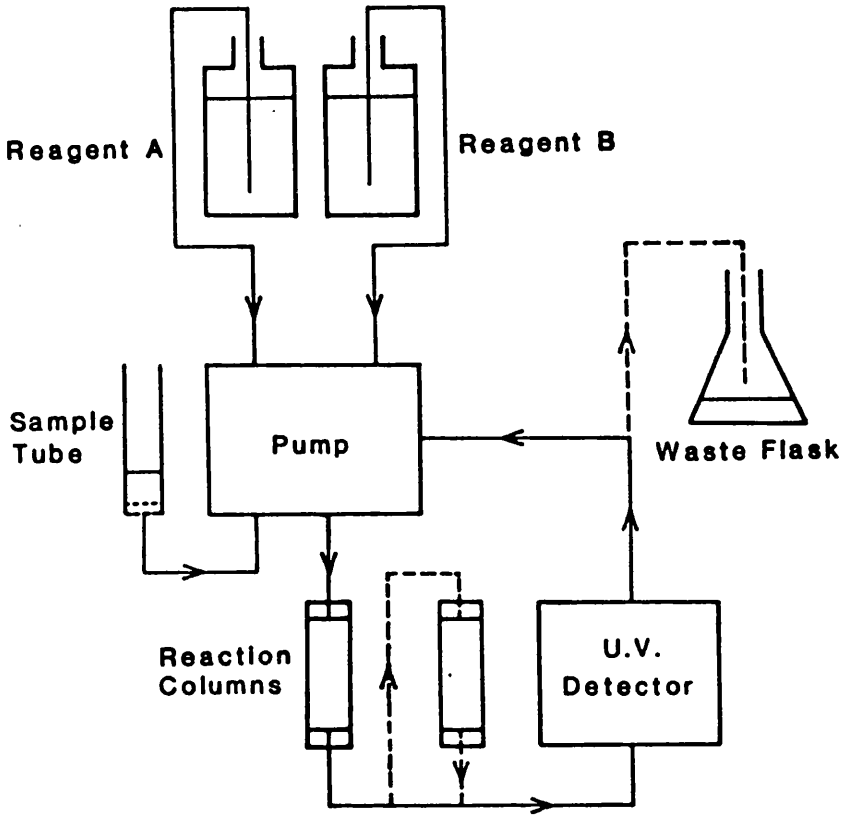
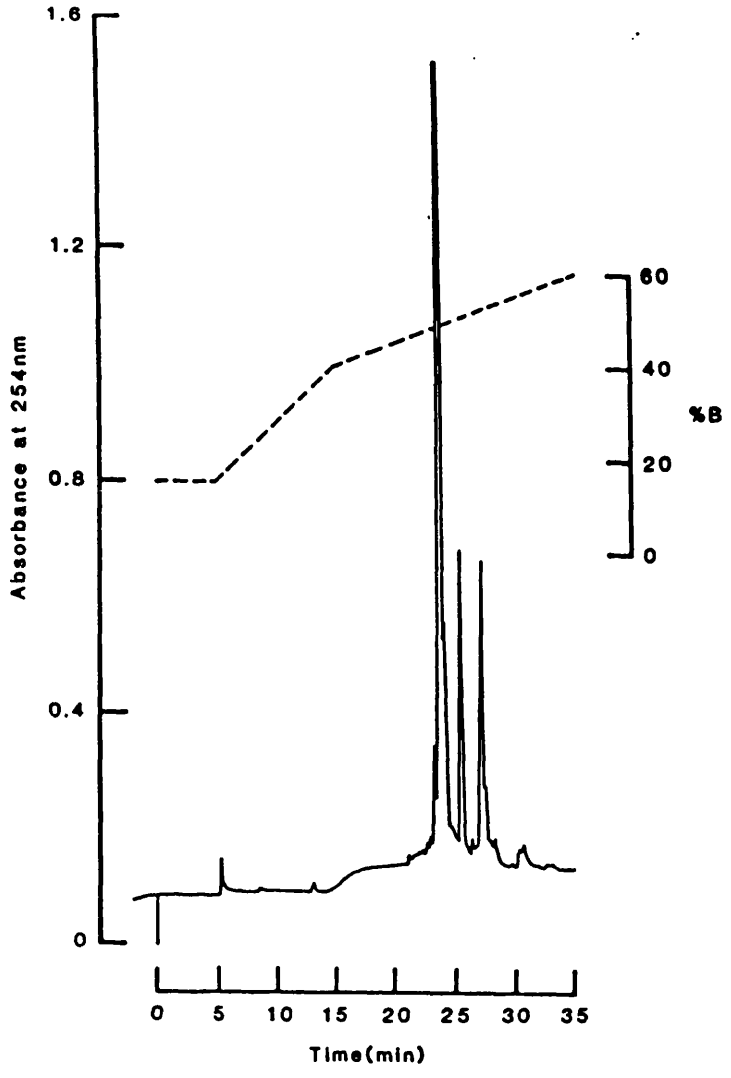


Fig. 2.1 Diagrammatic representation of the Pepsynthesiser II system. Reagent A is DMF; reagent B is 20% piperidine in DMF. Dotted lines indicate alternative flow routes.

Fig. 2.2. a) HPLC purification of synthetic M-AKH. A sample of crude peptide was loaded onto a Spherisorb 5 µm C_{18} column (30 cm x 0.8 cm) and eluted by a gradient indicated by the dotted line. Solvent A was 0.1% TFA; solvent B was 0.1% TFA in acetonitrile. The flow rate was 2 ml/min.

b) HPLC analysis of purified synthetic M-AKH. The sample was loaded onto a Spherisorb 5 µm C_{18} column (25 cm x 0.46 cm) and eluted by a gradient indicated by the dotted line. The solvents were the same as in a). The flow rate was 1 ml/min.


1.6
1.2
0.8
0.4
0
Absorbance at 254nm
60
40
20
0
%B
0 5 10 15 20 25 30 35
Time(min)

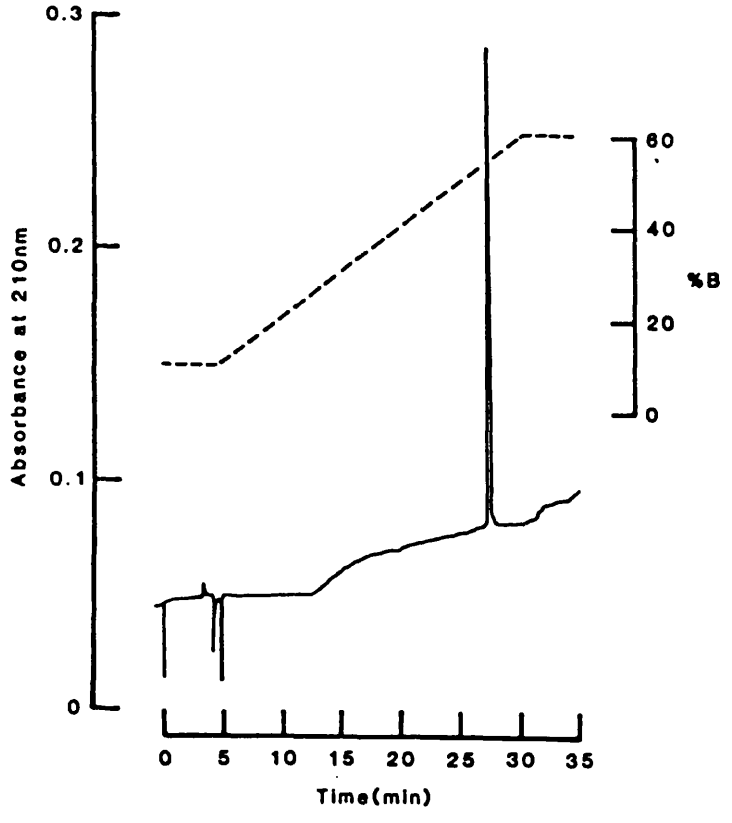
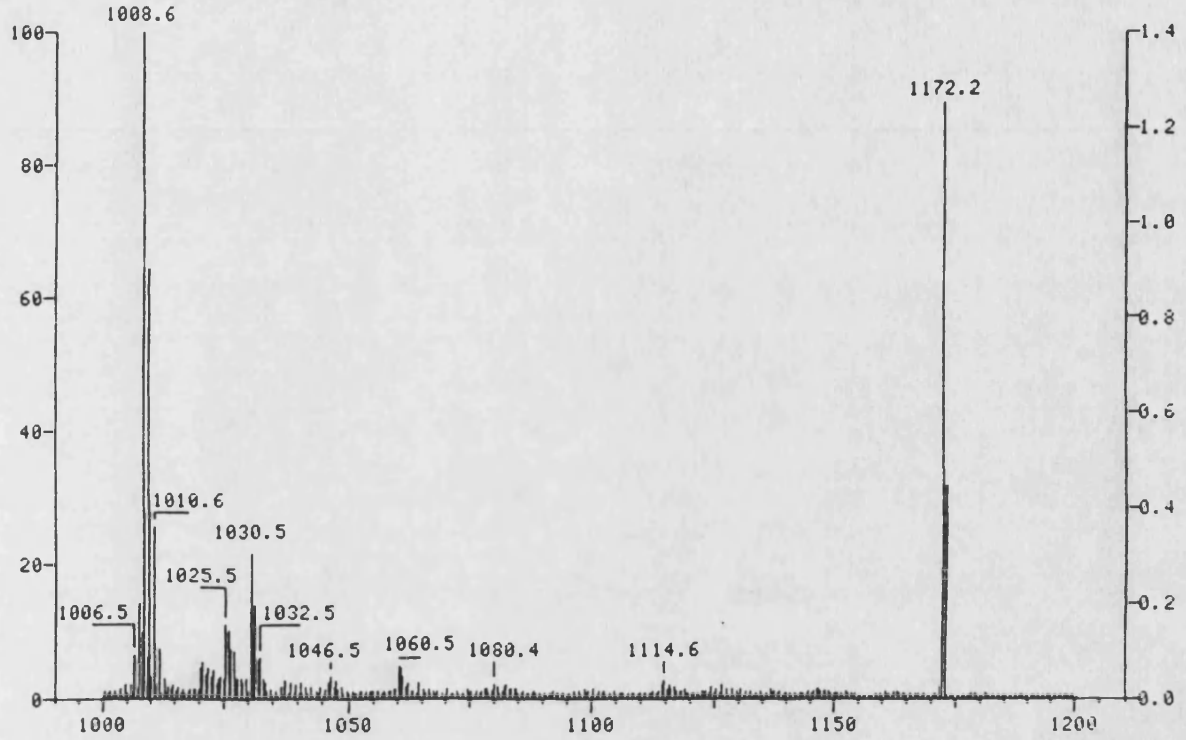

0.3
0.2
0.1
0
Absorbance at 210nm
60
40
20
0
%B
0 5 10 15 20 25 30 35
Time(min)

Fig. 2.3. a) Fast atom bombardment mass spectrometry (FAB-MS) of synthetic M-AKH. The peptide sample was loaded in a thioglycerol matrix and bombarded with Xenon gas in a Finnigan MAT-90 spectrometer. Mass to charge ratios (m/z) in the range 1000-1200 were scanned. Ion abundances are expressed in terms of absolute (right ordinate) and relative values (left ordinate).

b) Amino acid analysis of a sample of synthetic M-AKH, following hydrolysis for 24 h in 6 N HCl *in vacuo*. The analysis was performed by a Hilger Chromaspek II analyser. Ratios of amino acids are indicated relative to leucine.


1008.6
1172.2
1010.6
1030.5
1025.5
1006.5
1032.5
1046.5
1060.5
1080.4
1114.6
100
80
60
40
20
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1000
1050
1100
1150
1200

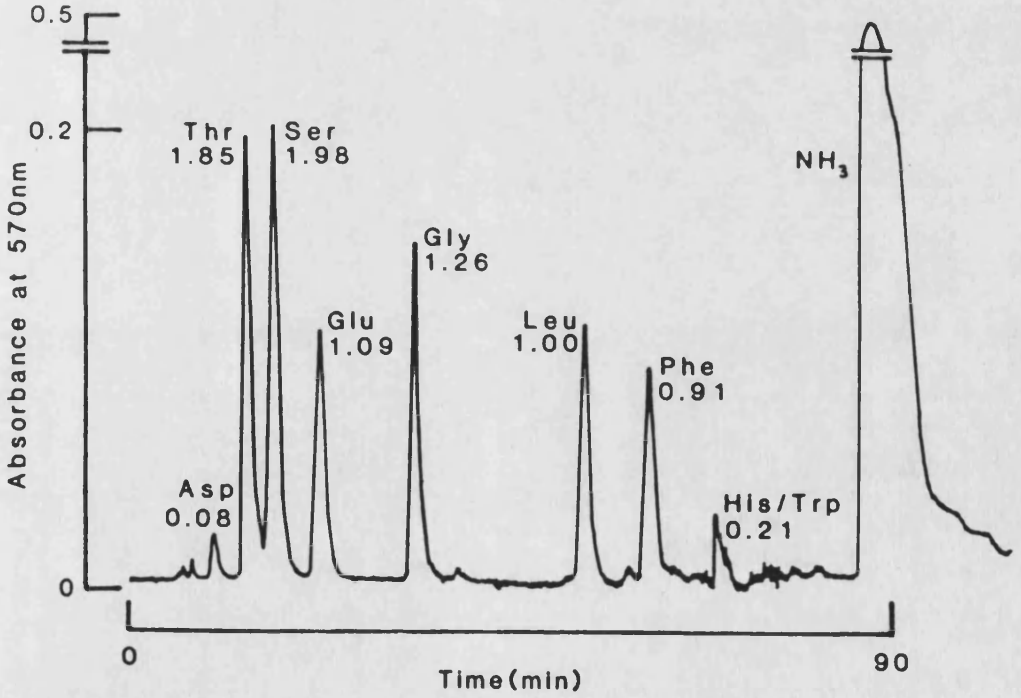
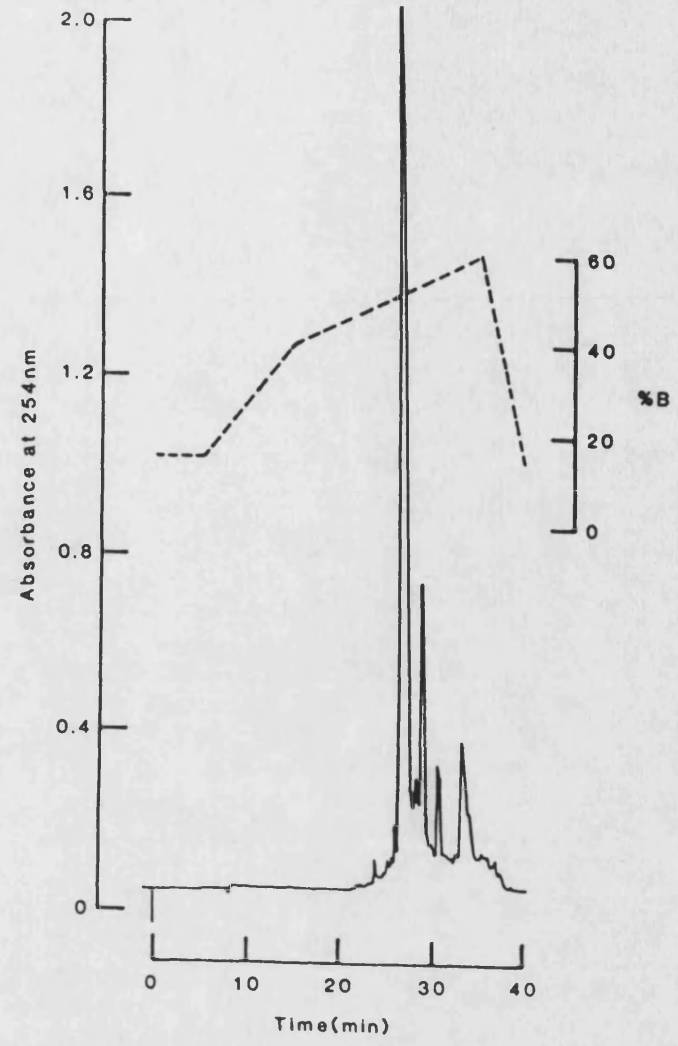

0.5
0.2
0
Absorbance at 570nm
Thr
1.85
Ser
1.98
Gly
1.26
Glu
1.09
Leu
1.00
Phe
0.91
NH3
Asp
0.08
His/Trp
0.21
0
90
Time(min)

Table 2.1. Amino acid analysis of the initial coupling step in the synthesis of the two analogues. The values were calculated from the peak heights of the glycine residue and the norleucine resin standard and calibrated from the values for a standard mixture of amino acids.

Resin Sample	Absolute Loading (meq/g)	
	Glycine	Norleucine
A	0.122	0.111
B	0.159	0.139
C	0.123	0.114
Mean	0.135	0.121

Fig. 2.4. HPLC purification (a) and analysis (b) of [Tyr^1]M-AKH. The chromatographic details were the same as in Fig. 2.2 except for the gradient used in b) indicated by the dotted line. For the analytical run a sample of [Tyr^1]M-AKH was loaded onto the analytical column together with synthetic M-AKH.


2.0
1.6
1.2
0.8
0.4
0
Absorbance at 254nm
60
40
20
0
%B
0
10
20
30
40
Time(min)

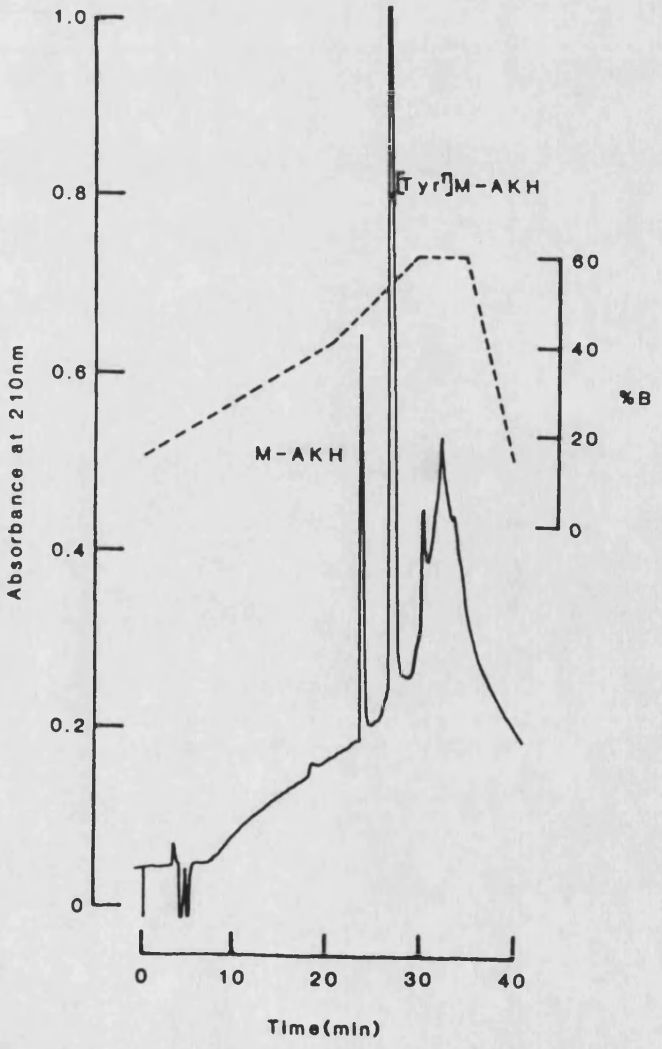
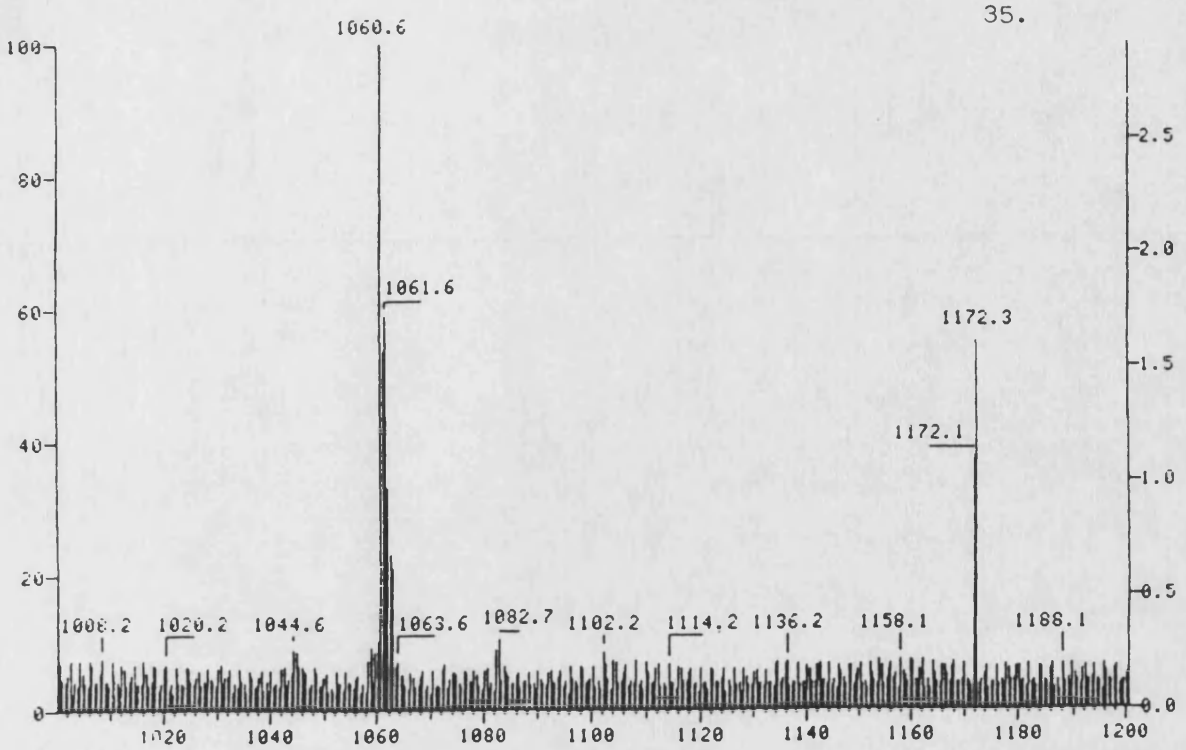

1.0
0.8
0.6
0.4
0.2
0
Absorbance at 210nm
[Tyr⁷]M-AKH
M-AKH
60
40
20
0
%B
0
10
20
30
40
Time(min)

Fig. 2.5. a) FAB-MS of [Tyr^1]M-AKH. Details as in Fig. 2.3.

b) Amino acid analysis of [Tyr^1]M-AKH. The hydrolysed sample was analysed with a norleucine reference sample. Other details were the same as in Fig. 2.3.


1060.6
1061.6
1172.3
1172.1
1008.2
1020.2
1044.6
1063.6
1082.7
1102.2
1114.2
1136.2
1158.1
1188.1
100
80
60
40
20
0
2.5
2.0
1.5
1.0
0.5
0.0
1020
1040
1060
1080
1100
1120
1140
1160
1180
1200

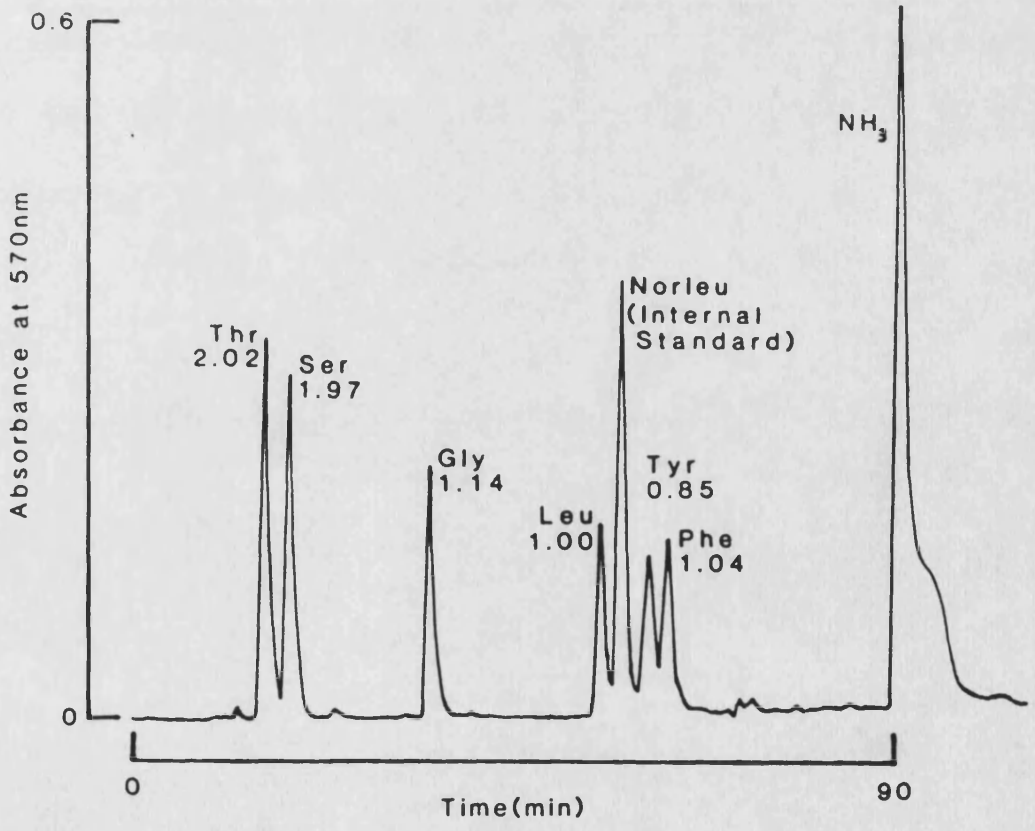
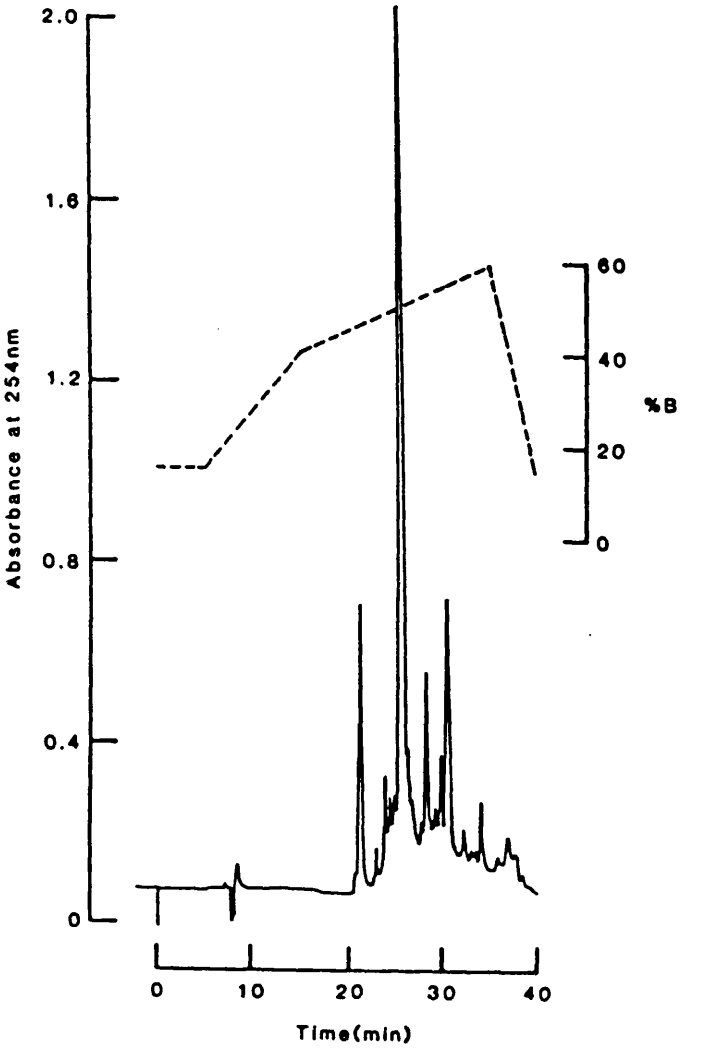

0.6
0
Absorbance at 570nm
Thr
2.02
Ser
1.97
Gly
1.14
Leu
1.00
Norleu
(Internal
Standard)
Tyr
0.85
Phe
1.04
NH3
0
90
Time(min)

Fig. 2.6. HPLC purification (a) and analysis (b) of [IodoPhe^4]M-AKH. Chromatographic conditions as in Fig. 2.2 except for the gradient used in b) indicated by the dotted line.


2.0
1.6
1.2
0.8
0.4
0
Absorbance at 254nm
60
40
20
0
%B
0
10
20
30
40
Time(min)

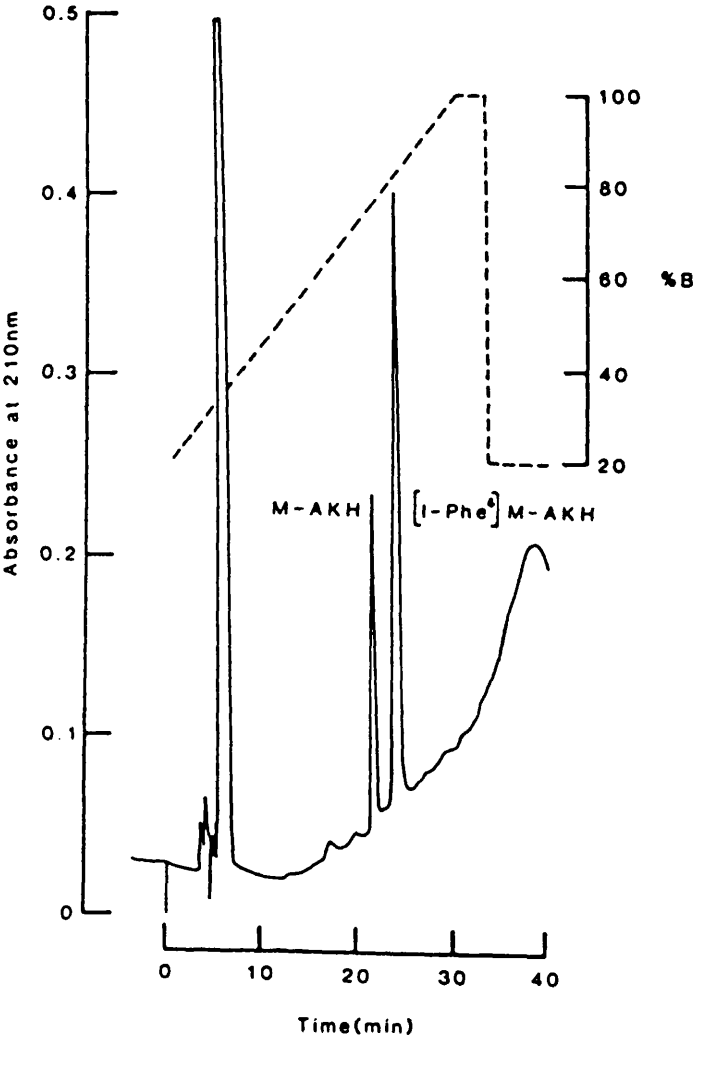
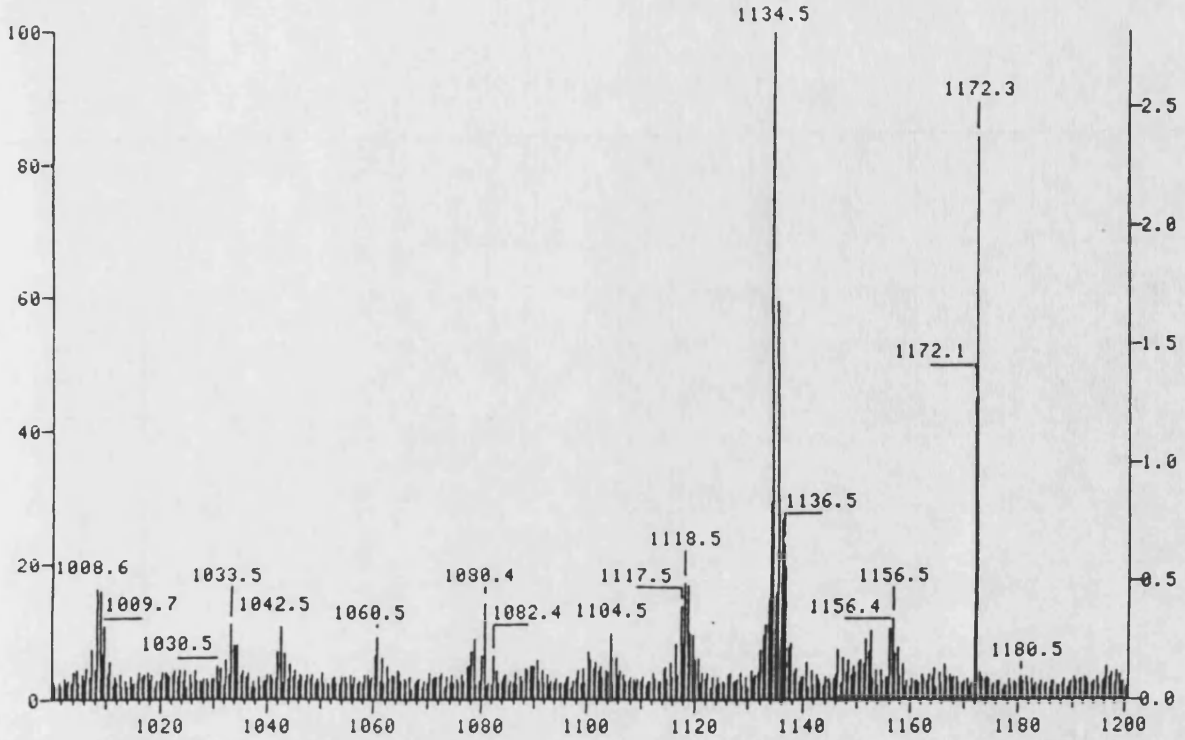

0.5
0.4
0.3
0.2
0.1
0
Absorbance at 210nm
100
80
60
40
20
%B
M-AKH
[I-Phe⁶] M-AKH
0
10
20
30
40
Time(min)

Fig. 2.7. a) FAB-MS of [IodoPhe^4]M-AKH. Details as in Fig. 2.3.

b) Amino acid analysis of [IodoPhe^4]M-AKH. The details were the same as in Fig. 2.5.


1134.5
1172.3
1172.1
1136.5
1118.5
1008.6
1033.5
1080.4
1117.5
1156.5
1009.7
1042.5
1060.5
1082.4
1104.5
1156.4
1030.5
1180.5
100
80
60
40
20
0
2.5
2.0
1.5
1.0
0.5
0.0
1020
1040
1060
1080
1100
1120
1140
1160
1180
1200

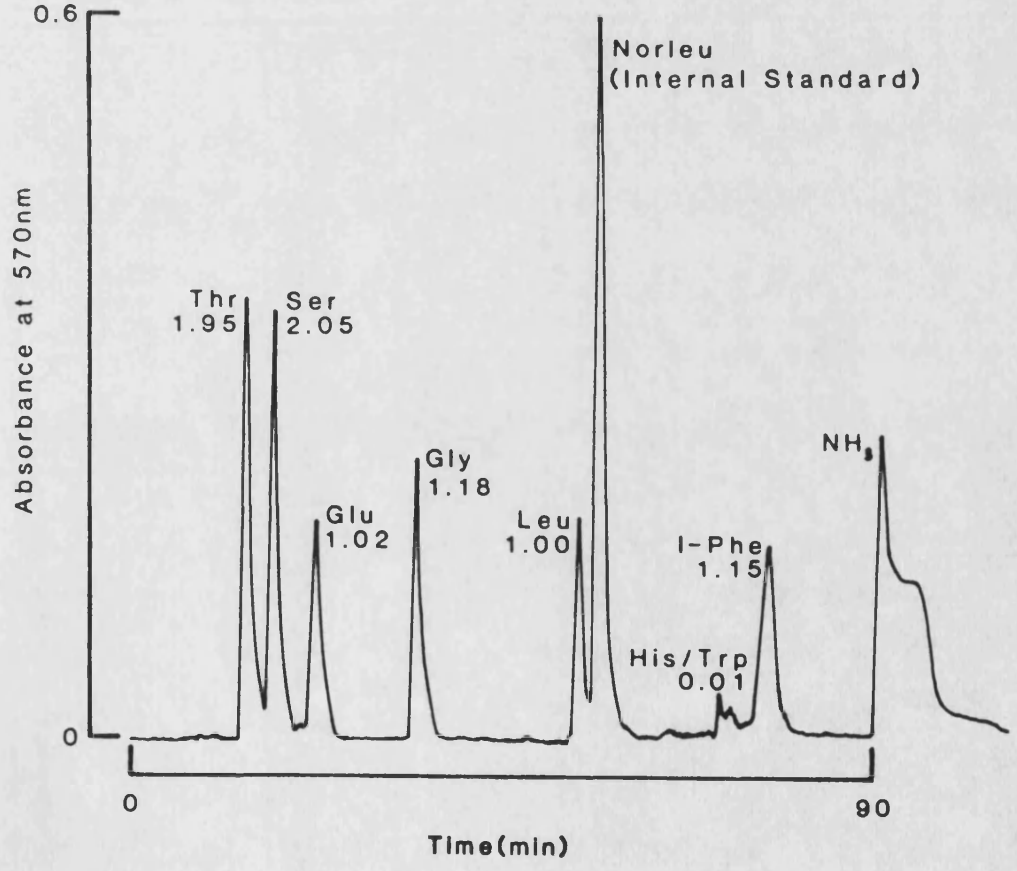

0.6
0
Absorbance at 570nm
Norleu
(Internal Standard)
Thr
1.95
Ser
2.05
Glu
1.02
Gly
1.18
Leu
1.00
NH3
I-Phe
1.15
His/Trp
0.01
0
90
Time(min)

Table 2.2. Adipokinetic activity of synthetic M-AKH and analogues in adult *Manduca*.

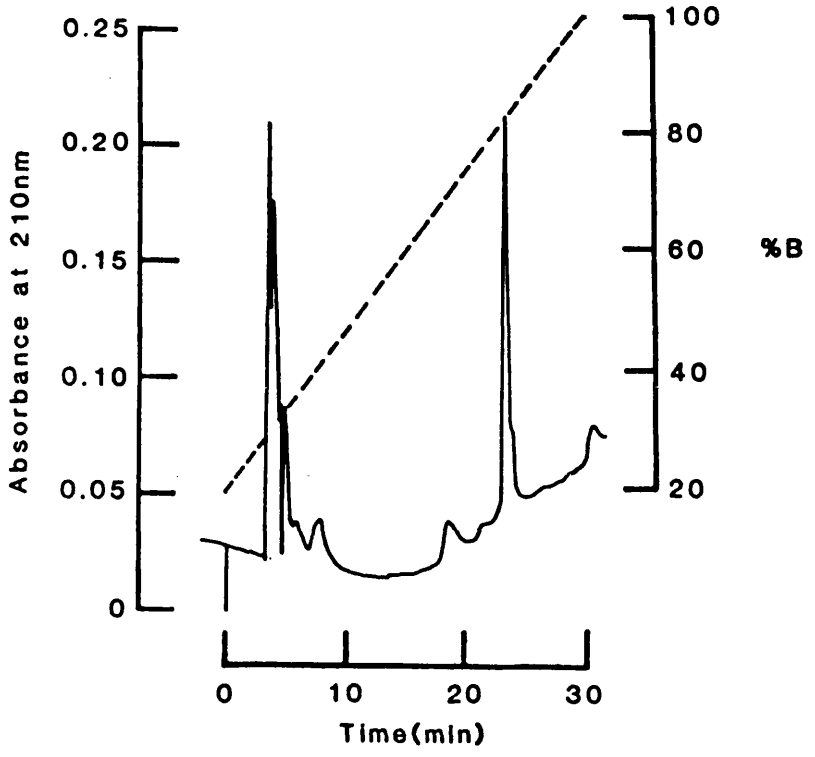
Peptide	Dose (pmol)	Increase in Haemolymph Lipids (mg/ml)	n
M-AKH	2.0	33.0 ± 2.0*	4
	10.0	33.5 ± 7.5*	4
[IodoPhe^4]M-AKH	5.0	7.9 ± 1.8	8
	20.0	17.1 ± 3.0*	7
[Tyr^1]M-AKH	20.0	5.9 ± 1.8	7

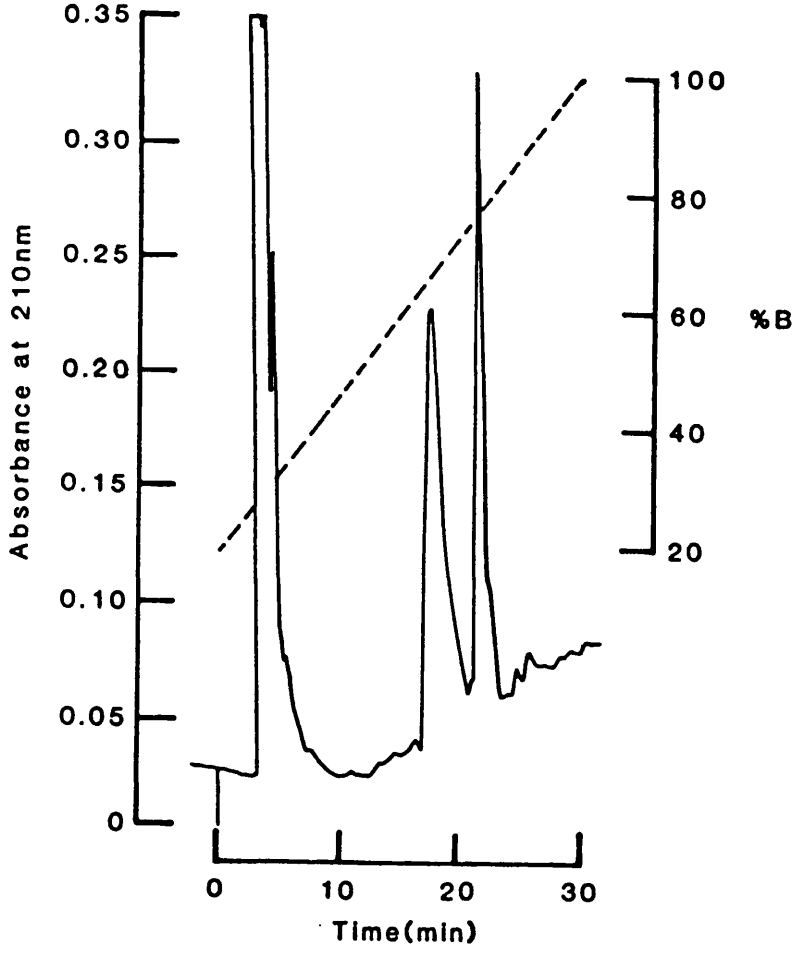
Response expressed as the mean ± SE for n samples. Asterisks indicate a significant increase compared with control injections of 10% acetonitrile (5.5 ± 1.0, n = 4) as determined by a t-test ($p < 0.01$).

Fig. 2.8. Trial hydrogenation of [IodoPhe^4]M-AKH.

a) Control. HPLC analysis of peptide following exposure to $Pd/CaCO_3$ catalyst in DMF under a normal atmosphere. The major peak coeluted with [IodoPhe^4]M-AKH.

b) Experimental. HPLC analysis of peptide following exposure to $Pd/CaCO_3$ catalyst in DMF perfused with hydrogen gas. The major peak coeluted with M-AKH.


0.25
0.20
0.15
0.10
0.05
0
Absorbance at 210nm
100
80
60
40
20
%B
0
10
20
30
Time(min)


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
Absorbance at 210nm
100
80
60
40
20
%B
0
10
20
30
Time(min)

CHAPTER 3. A RADIOIMMUNOASSAY FOR M-AKH AND ITS USE IN M-AKH QUANTIFICATION

INTRODUCTION

The amount of peptide hormone contained within a tissue may be determined by a variety of techniques, including bioassay, chromatography (particularly reversed-phase HPLC) and radioimmunoassay (RIA). Some of these techniques have been used for the determination of the AKH-I content of tissues, principally the corpora cardiaca (CC), from locusts. Stone et al. (1976) calculated the recovery of AKH-I from Locusta and Schistocerca from the amino acid content of tissue extracts and used this information to estimate the AKH-I content of Locusta and Schistocerca CC. Van Marrewijk et al. (1983) compared the bioactivity of CC extracts with that of synthetic AKH-I to obtain an estimate of the AKH-I content of Locusta CC. The most extensive quantification to date, was performed by Siegert and Mordue (1986) using reversed-phase HPLC. They determined the AKH-I and AKH-II content of CC from male and female locusts during the last larval instar and during adulthood.

Peptides may be quantified by radioimmunoassay at levels which cannot be detected by other techniques. Radioimmunoassays have been developed for the investigation of AKH-I biosynthesis in the CC (Hekimi and O'Shea, 1987) and in the brain (Moshitzky et al., 1987a, 1987b) but only one of these studies included any data on the AKH-I content of CC and brain (Moshitzky et al., 1987a).

I have raised an antiserum for M-AKH and have used it in the development of a specific RIA for M-AKH. The RIA has been used to determine the M-AKH content of larval and adult CC and neural tissue from Manduca. The results will be compared with RP-HPLC data for the M-AKH content of adult CC.

MATERIALS AND METHODS

Experimental Animals

The animals used in this project were from a laboratory culture of Manduca sexta maintained on artificial diet according to Bell and Joachim (1976), under a long day (LD, 17:7) photoperiodic regime.

Preparation of Tissue Extracts

Brain, abdominal nerve cord and CC extracts were prepared from adult and larval tissues. Adult tissues were obtained from day 2 moths of both sexes (which are of similar size). Larval tissues were obtained from day 3, fifth instar larvae (also unsexed). The extraction method was slightly modified from that reported by Jaffe et al. (1986) for the extraction of the adipokinetic hormone from the corn earworm, Heliothis zea.

Tissues were excised, rinsed briefly in insect saline (Ephrussi and Beadle, 1936), blotted lightly on filter paper and placed in 1.5 ml polypropylene tubes on ice. Each tissue sample was homogenised in 50-200 µl of extraction solvent (methanol : water : glacial acetic acid, 90:9:1) in a small glass homogenizer and then centrifuged at 12,000 r.p.m. for 5 min in an Eppendorf 5415 micro-

centrifuge. The supernatant was removed and the pellet reprocessed through the homogenization and centrifugation steps. Pooled supernatants were freeze-dried in a Speed Vac concentrator and resuspended in 1 ml of 0.1% TFA. Ethyl acetate (3 x 500 µl) was mixed with the TFA solution to extract any lipids from the sample. The ethyl acetate fraction separated out above the TFA solution and was removed with a pipette. Any residual ethyl acetate was removed under nitrogen and the sample was then applied to a C_{18} SepPak cartridge primed with 10% acetonitrile. The sample was washed with 10% acetonitrile and eluted by a 60% acetonitrile wash. The eluate was lyophilized and resuspended in 10% acetonitrile or RIA buffer for HPLC or RIA titre determination.

Haemolymph M-AKH Titre Determination

Haemolymph M-AKH titres were determined in starved and fed larvae. All larvae were manually removed from the artificial diet on which they were reared. The control insects were immediately replaced on diet, whereas the experimental insects were placed in empty plastic cups. The larvae were then placed in an incubator at 25°C for different lengths of time prior to haemolymph extraction. Any faecal pellets produced during the experiment by the starved larvae were removed to prevent coprophagy.

Larvae were rapidly anaesthetized with CO_2 and then bled by removal of the abdominal horn. 1 ml of haemolymph was collected from each larva in a polypropylene tube containing a few crystals of P.T.U. Each sample was rapidly frozen in dry ice/ethanol and then placed in a boiling water bath for 3 min. The freezing step

was included to prevent the enzymatic degradation of any released peptide by haemolymph proteases (see Chapter 6) and the boiling step precipitated haemolymph proteins. Boiled samples were centrifuged at 2,000 r.p.m. for 5 min in Beckman J-6 centrifuge and the supernatant was then removed and applied onto a primed C_{18} SepPak cartridge. The material eluting between 10% and 60% acetonitrile was collected and lyophilised. Each haemolymph extract was resuspended in 50 µl of RIA incubation buffer and assayed.

HPLC Analyses

The M-AKH titre of adult CC extract was determined by RP-HPLC using the Gilson system described in the previous chapter. A Spherisorb C_{18} 5 µm analytical column (0.46 x 25 cm, HPLC Technology) was used for the quantification. The flow rate was 1 ml/min and the solvents used were: A, 0.1% TFA; B, 0.1% TFA in 60% acetonitrile. The gradient conditions for the quantification were: 33-77.5% B (0-20 min), 77.5-100% B (20-20.1 min), 100% B (20.1-25 min), 100-33% B (25-25.1 min) followed by a reequilibration period of 15 min at 33% B. The eluate was monitored by u.v. absorbance at 210 nm. Peak areas were calculated by a Gilson Model 620 Data Master. Synthetic M-AKH standards were used to calibrate the system and to indicate the elution time for M-AKH under the above conditions.

In a subsequent experiment to determine the elution time of the M-AKH-immunoreactive material, a sample of CC or brain extract was injected onto a Spherisorb C_{18} 5 µm column (as above). The solvents and flow rate were the same as those described above, however the

sample was eluted by a linear gradient of 20-90% solvent B over 40 min. Fractions were collected in 4 ml polypropylene tubes at 1 min intervals using a Gilson Model 201-202 fraction collector. An aliquot from each fraction was removed, lyophilised and then resuspended in RIA buffer (aliquots outside the region of interest were pooled prior to lyophilisation).

Preparation of Radiolabelled [Tyr^1]M-AKH

1 µg of [Tyr^1]M-AKH was radioiodinated by a chloramine-T method previously used for the iodination of the trout melanophore concentrating hormone, MCH (Eberle, 1987). The iodination procedure was performed in a fume cupboard specifically designated for that purpose. 10 µl of Na^{125}I (1 mCi, Amersham International, Amersham, U.K.) was pipetted into a 1.5 ml polypropylene tube and stored in a lead castle. 20 µl of 0.25 M phosphate buffer, pH 7.4 was added to the isotope followed by 1 µg of [Tyr^1]M-AKH in 10 µl of 10% acetonitrile and 15 µl of chloramine-T solution (5 mg/10 ml of 0.25 M phosphate buffer, pH 7.4 prepared immediately before use). The iodination cocktail was mixed immediately and then left to stand for 30 sec. Termination of the iodination was achieved by the addition of 600 µl of 0.05 M phosphate buffer, pH 7.4 containing 0.25% BSA and 0.1% ß-mercaptoethanol.

A SepPak protocol was used to separate the labelled peptide from free Na^{125}I (it was assumed that all the unlabelled peptide had been iodinated by the large excess of isotope). A C_{18} SepPak cartridge had been primed, before the iodination, by washes of 1% TFA (3 x 600 µl); 80% methanol (3 x 600 µl); 20 mg/2 ml polypep

(from Sigma) in 0.05 M phosphate buffer, pH 7.4 (1 x 600 µl); 80% methanol (3 x 600 µl) and 1% TFA (3 x 600 µl). The iodination mixture was applied to the primed SepPak and the eluate was reapplied and collected. The SepPak was then washed with 0.25 M phosphate buffer (2 x 600 µl) followed by a graded series of methanol solutions in 1% TFA, from 30% to 80% methanol with 5% intervals between each concentration. For each concentration the SepPak was washed three times with 600 µl of solution and the eluates were collected separately in polyproylene tubes. A 10 µl aliquot from each tube was counted for 6 sec in a Type N550A scintillation counter (Ecko Electronics Ltd., Southend, U.K.) to determine which fractions contained the labelled peptide. The peptide-containing fractions were diluted with 3 ml of 50% methanol and 3 ml of termination buffer and stored in sealed polypropylene tubes at -20°C.

Conjugation of [Tyr^1]M-AKH to Thyroglobulin

Two conjugation procedures were employed using different linking agents to conjugate the peptide to bovine thyroglobulin.

Method A. 2 mg (2 µmol) of [Tyr^1]M-AKH was dissolved in 1.5 ml of 0.1 M phosphate buffer, pH 7.5, with the addition of a few drops of DMF to ensure complete dissolution. 50 mg of thyroglobulin was added to the peptide solution and the mixture placed on ice. 1.6 ml of 0.02 M glutaraldehyde in 0.1 M phosphate buffer, pH 7.5 was added in 300 µl aliquots over a 15 min period. The mixture was shaken regularly during this period and then left to incubate

overnight at 4°C The conjugated mixture was dialysed for 24 h against 0.1 M phosphate buffer, pH 7.4 and against distilled water for a further 24 h. The dialysed conjugate was then lyophilised and stored in a desiccator at -20°C. The estimated molar ratio of protein : peptide : glutaraldehyde was 0.037:1:16, therefore 1 mg of peptide should have been present in 25.9 mg of conjugate.

Method B. This procedure is based upon that used by Schooneveld et al. (1983) for the conjugation of [Tyr^1]AKH-I to thyroglobulin. 1.8 mg (4 µmol) of bis(2-[succinimidooxycarbonyloxy]ethyl)-sulphone (BSOCOES, purchased from Pierce U.K. Ltd., Cambridge, U.K.) was dissolved in 120 µl of DMF together with 2 mg (2 µmol) of [Tyr^1]M-AKH. After 2 min this mixture was added to 40 mg of thyroglobulin in 1.2 ml of 0.01 M phosphate buffer, pH 7.4. The mixture was incubated at room temperature for 75 min and then dialysed overnight against distilled water. The dialysed material was then lyophilised and stored in a desiccator at -20°C. The estimated molar ratio of protein : peptide : BSOCOES was 0.03:1:2 therefore 1 mg of peptide should have been present in 20.6 mg of conjugate.

Immunization Protocol

Two male Sandy half-lop rabbits were injected with conjugated peptide. 5 mg of conjugate (approximately 200 µg of peptide equivalent) was dissolved in 1 ml of 0.05 M phosphate buffered saline, pH 7.2, emulsified in 2 ml of complete Freund's adjuvant (Sigma) and injected into each rabbit at multiple sites

intramuscularly. The primary immunization was repeated two weeks later and booster injections of 180-200 µg of peptide equivalent, emulsified in 2:1 incomplete Freund's adjuvant : phosphate buffered saline were administered at fortnightly intervals. Serum samples were obtained by ear-bleeding the rabbits 8-10 days after each injection.

Peptide conjugated according to Method A was used for the primary immunizations and earlier boosts. When the supply of this conjugate was exhausted Method B conjugate was administered. The anti-M-AKH response was assessed by the ability of serum to precipitate [^{125}I-Tyr^{1}]M-AKH. When the titre of antibody was suitably high the rabbits were anaesthetised with halothane and exsanguinated by cardiac bleeding. The blood was collected in pyrex beakers and left to stand at room temperature for 2 h. The coagulated blood was then placed at 4°C to encourage contraction of the clot. The final serum was divided into 1 ml aliquots and stored at -70°C.

Radioimmunoassay for M-AKH

A radioimmunoassay was developed by the modification of protocols previously developed for the assay of MCH (Dr B.I. Baker, personal communication) and AKH-I (Moshitzky et al., 1987a). The incubation buffer used for the dilution of the serum, labelled peptide and test samples consisted of 0.05 M phosphate buffer pH 7.4 containing 0.25% BSA, 0.01% thiomersal and 0.001% poly-L-lysine (m.wt. 4,000-15,000). The incubation mixture contained 50 µl of buffer (or sample in later experiments), 50 µl

of diluted serum and 50 µl of labelled peptide (9,000 c.p.m.). In order to assess the antibody titre each serum sample was serially diluted in incubation buffer. The RIA mixture was incubated overnight at 4°C and terminated by the addition of 500 µl of charcoal solution (0.05 M phosphate buffered saline containing 0.1% gelatine, 0.125% dextran, 0.01% thiomersal and 0.5% activated charcoal, pH 7.4 at 4°C). The terminated mixture was incubated at 4°C for 7-8 min and centrifuged at 4,500 r.p.m. for 15 min in a Beckman J-6 centrifuge. The supernatant containing labelled peptide bound to antibody was aspirated from each tube and the pellet, containing free labelled peptide bound to charcoal, was counted in an LKB 1275 Minigamma counter.

All assays included controls lacking antibody or unlabelled peptide. In order to test the specificity of the assay, a number of other insect peptides were assayed for cross-reactivity. These were obtained from the following sources: AKH-I from Peninsula Laboratories, St. Helens, U.K.; AKH-II(L) from Professor W. Mordue, University of Aberdenn, U.K.; M-II from Professor M. O'Shea, University of Geneva, Switzerland; HTF-II from Dr. G. Gäde, University of Dusseldorf, FRG; Leucokinins II and VI (LKII and LKVI) from Dr. G.M. Holman, U.S. Dept. of Agriculture, College Station, U.S.A.

RESULTS

The M-AKH content of adult and larval tissues was determined by HPLC and RIA analysis. The contents of less than 10 adult CC pair equivalents were detectable by u.v. absorbance of the HPLC eluate

(Fig. 3.1a). A single major peak was observed which coeluted with a sample of synthetic M-AKH (Fig. 3.1b). A standard curve was obtained for the peak areas of varying amounts of synthetic M-AKH injected in 100 µl of 10% acetonitrile (Fig. 3.2). Two samples containing 3.9 and 7.8 CC pair equivalents were analysed and the peak areas indicated titres of 19.0 and 19.9 ng/CC respectively. The M-AKH content of larval CC extract was not detectable using similar quantities of extract.

A radioimmunoassay was developed to provide a more sensitive assay for the detection of M-AKH in larval and adult tissues. The results of the radioiodination of [Tyr^1] M-AKH are illustrated in Fig. 3.3. The majority of the radioactivity (free $Na^{125}I$) was eluted from the SepPak by the initial washes. Another peak of radioactivity was eluted by the 55% methanol washes. This peak represented the radiolabelled M-AKH analogue and fraction 55_2 was used in the RIA experiments.

The first attempt to raise antibodies to M-AKH utilised conjugate produced by Method B. This material was injected into three Californian White rabbits but failed to evoke a suitable response. The failure of this attempt may have been due to an underestimate of the amount of conjugate required for each injection, based on the molar ratio quoted by Schooneveld *et al.* (1983). A second attempt was made using Sandy half-lop rabbits initially injected with conjugate produced by Method A and subsequently with the Method B conjugate. This was more successful and Fig. 3.4a shows the titre of antibody (represented as the % of labelled peptide bound to antibody) from the serum of rabbit number

158 following the terminal bleed. The maximum binding observed was only 40%, however there was sufficient immunoreactivity for the development of an RIA. A final serum dilution of 1 in 1800 was used in subsequent experiments. The sensitivity and specificity of the RIA are illustrated in Fig. 3.5b. The displacement of bound labelled peptide by unlabelled M-AKH indicated that the assay could be used for the detection of 40-400 pg of M-AKH. A number of other insect peptides were also assayed for cross-reactivity with the M-AKH antiserum. Several members of the AKH/RPCH family (AKH-I, AKH-II(L), M-II and HTF-II) failed to displace the labelled M-AKH analogue at any concentration tested. However Leucokinin II (LKII, Asp-Pro-Gly-Phe-Ser-Ser-Trp-GlyNH_2, Holman et al., 1986) a cockroach peptide with the same C-terminal sequence as M-AKH, caused a modest displacement of the label from the antibody but only at a very high concentration (5 ng of LK-II per tube).

The M-AKH RIA was used to determine the amount of M-AKH in brain, nerve cord and CC extracts prepared from larval and adult Manduca (Table 3.1). The RIA confirmed the HPLC quantification of M-AKH in adult CC. The content of the adult CC (21 ng/CC) is ten times greater than the content determined for larval CC (2.2 ng/CC). Much smaller quantities of M-AKH immunoreactivity were detected in the adult brain and abdominal nerve cord extracts (0.67 and 0.08 ng/tissue, respectively) and in the larval brain and nerve cord extracts (0.06 and 0.11 ng/tissue, respectively).

The majority of the immunoreactivity in adult and larval CC extracts was contained in the fraction eluting between 25 and 26 min (Fig. 3.6b). For the adult CC extract this peak of

immunoreactivity corresponded to a u.v.-absorbing peak which co-eluted with synthetic M-AKH (Fig. 3.6a). However both extracts also display a smaller amount of immunoreactivity in the pooled fractions which eluted between 30 and 34 min. There was no u.v.-absorbing peak in this region. A u.v. peak at 39 min did not correspond to M-AKH like immunoreactivity. Comparison with blank HPLC runs, when no sample was injected, suggested that this peak was probably due to a solvent contaminant.

The adult and larval brain extracts did not possess immunoreactive material which co-eluted with M-AKH, but they did possess some immunoreactive material in the 30-34 min pooled fractions (data not shown).

Haemolymph titres of M-AKH in starved and fed larvae were measured using the RIA procedure. M-AKH immunoreactive material was released into the haemolymph during the first hour of larval starvation (Fig. 3.7). The haemolymph concentration of M-AKH increased from approximately 120 pg/ml to 300 pg/ml and then returned to a resting level of approximately 100 pg/ml within 3 h of the onset of starvation. These changes in the haemolymph M-AKH concentration would require the secretion into the haemolymph of about 180 pg of M-AKH since the haemolymph volume of a day 3 larva is about 1 ml. A slight increase in the titre of immunoreactive material in the control (fed) larvae during the first hour of the experiment may have been a result of handling at the start of the experiment.

DISCUSSION

In locusts, AKH-I is synthesised and secreted by intrinsic

neurosecretory cells within the glandular lobe of the CC (Orchard, 1987). Stone et al. (1976) estimated the titre of AKH-I in the CC to be 200-500 pmol in Locusta and 400-700 pmol in Schistocerca CC. More recently Siegert and Mordue (1986) used RP-HPLC to determine the titres of AKH-I and AKH-II in the CC of Schistocerca and Locusta. They confirmed that Schistocerca CC contain more peptide than those of Locusta and reported an increasing amount of both peptides from the last larval instar through to adulthood. The AKH-I content increased from 100-750 pmol/CC in Locusta and from 200-1200 pmol/CC in Schistocerca. The ratio of AKH-I to AKH-II increased from 2:1 in fifth instar Locusta to 6:1 in adult Locusta. Female locusts (which are larger than males) generally possessed higher amounts of both peptides than males.

Ziegler et al. (1984) used a bioassay to estimate that the CC of adult Manduca contained 10-20 times less adipokinetic peptide than adult locust CC. The synthesis, storage and release of glycogen phosphorylase activating hormone (GPAH), a peptide which may be identical to M-AKH (see Chapter 4), has recently been demonstrated within the intrinsic neurosecretory cells of the larval CC (Ziegler et al., 1988). The data presented here confirm both the earlier estimate of the M-AKH content of Manduca CC and the importance of the CC as the primary if not the only source of M-AKH. As in the locust, a considerable increase in the hormone content was observed between the last larval instar and adulthood (2.2-21 pmol/CC). This may reflect an increased demand for the hormone as a regulator of lipid metabolism during flight (Ziegler and Schulz, 1986a). The lower amount of hormone in adult Manduca

compared with the amount of AKH-I in the adult locust probably reflects differences between the species in the amount of time spent in sustained flight as well as the greater longevity of the adult locust (several weeks) compared with the adult moth (approximately 1 week).

HPLC analysis of the CC extracts indicated that the majority of the immunoreactive material coeluted with M-AKH. This confirmed the specificity of the RIA for M-AKH, however there did appear to be some cross-reactivity with non-M-AKH material. Both the larval and adult CC extracts contained immunoreactive material which eluted later than M-AKH from the HPLC column (30-34 min). This material may represent a precursor polypeptide or a second AKH-family peptide. The latter explanation would be consistent with the presence of a second peptide in the CC of other insect species, in particular a second peptide recently isolated from the CC of the corn earworm moth Heliothis zea (Jaffe et al., 1988b). The identification of the unknown material will depend upon further HPLC purification and bioassay of the fractions eluted between 30 and 34 min.

The presence of AKH and AKH-like peptides in the insect brain has been the subject of some speculation. Schooneveld et al. (1983) using a 'C-terminal specific' antiserum for AKH-I demonstrated immunoreactive neurones in the locust brain. A subsequent study using an 'N-terminal specific' antiserum for AKH-I suggested that the AKH-like material in the locust brain was not AKH-I (Schooneveld et al., 1986). An RIA for AKH-I has recently been employed to identify two AKH-like factors in the locust brain

(Moshitzky et al., 1987a). One of these factors has been identified as AKH-I on the basis of bioassay and RP-HPLC data and its synthesis in the brain has been demonstrated (Moshitzky et al., 1987b). The physiological role of locust brain AKH-I is not known.

The amounts of immunoreactive material determined from Manduca brain and nerve cord extracts were much lower than from the CC. HPLC analysis of the brain extracts indicated the absence of M-AKH from larval and adult brains, however the 30-34 min peak of immunoreactive material observed in the CC was also apparent in the brain extracts. Therefore the immunoreactive material quantified in the brain and nerve cord extracts was probably not M-AKH. Brain extracts from larval and adult Manduca have been shown to lack glycogen phosphorylase activating activity (Ziegler, 1979; Ziegler et al., 1988), but the adipokinetic activity of brain extracts has yet to be determined. The identification of the unknown immunoreactive material in terms of both its structure and its biological activity will be an important goal for future work.

The M-AKH immunoreactive material released into the haemolymph of starved larvae has not been further characterised in terms of its HPLC retention time or biological activity. However the data from the other tissue extracts and the cross-reactivity experiment (Fig. 3.5) suggest that the RIA is relatively specific for M-AKH. It would therefore seem reasonable to assume that the immunoreactive material quantified in Fig. 3.7 represents M-AKH in the haemolymph.

If M-AKH is the same as the larval glycogen phosphorylase activating hormone (Ziegler et al., 1987), the M-AKH titre in the

haemolymph of 'resting' larvae was unexpectedly high, apparently enough to cause significant activation of larval phosphorylase (see Chapter 4). Actually the levels of active phosphorylase in such insects are known to be low (Siegert, 1987). The explanation for this apparent contradiction may lie in the dynamics of M-AKH release, distribution and inactivation. First, it is distinctly possible that M-AKH may be rapidly released when the larvae are anaesthetised and bled. I have found that 'resting' levels of active phosphorylase are high unless great care is taken to minimise the trauma of killing and dissecting the insects and the time taken for these procedures. Second, the circulation of haemolymph in larval Manduca is known to be slow compared with the rapidity of the fat body phosphorylase response to injected CC extract (Ziegler, 1979). Consequently a dose of 100 pg of M-AKH injected into a larval abdomen (the location of the majority of the fat body tissue) may be considerably more potent than 100 pg of M-AKH dispersed throughout the haemolymph. Third, the activities of M-AKH inactivating enzymes in the haemolymph (and possibly in other tissues) may result in the non-uniform distribution of M-AKH activity throughout the haemocoel (see Chapter 6).

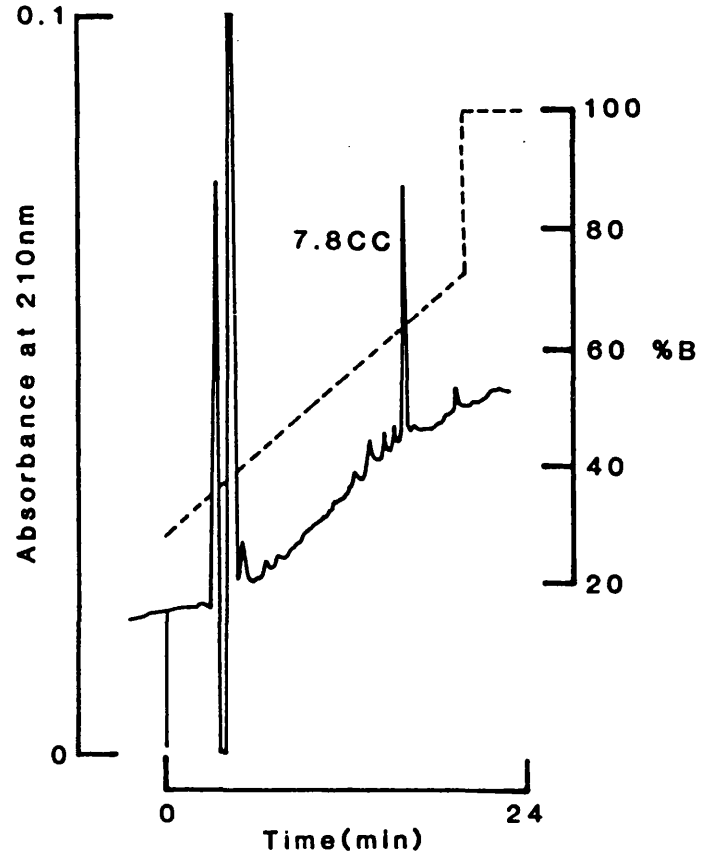
The physiological significance of M-AKH release into the haemolymph during the first hour of starvation will be discussed in the next chapter. The immunoreactive M-AKH titre declined during the second and third hours of starvation indicating the removal of M-AKH from the haemolymph, possibly by enzymatic degradation (see Chapter 6). The activity of fat body glycogen phosphorylase itself appears to decline more slowly, since Siegert (1987) reported a

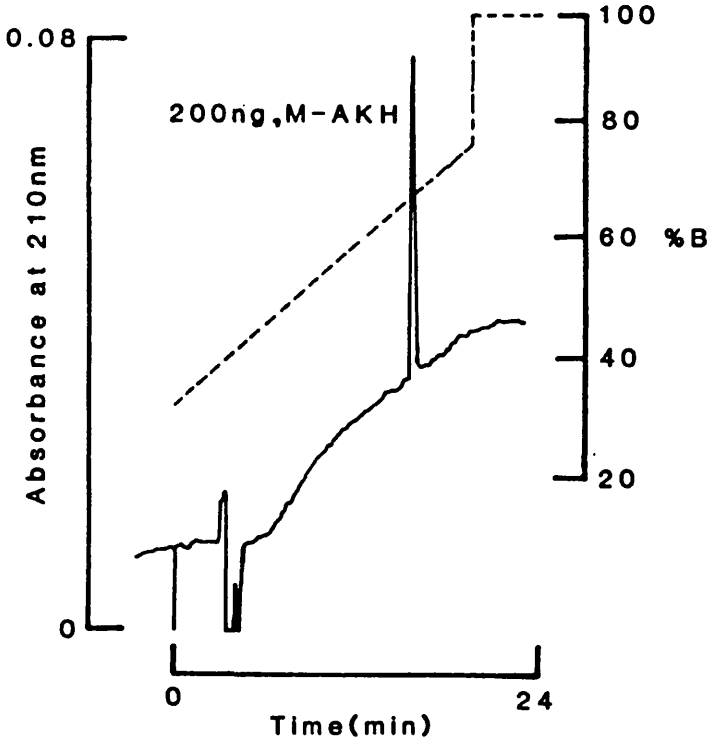
return to resting levels only after 48 h. My data support the suggestion made by Siegert (1988) that phosphorylase inactivation is facilitated by the removal of M-AKH from the haemolymph rather than a change in the responsiveness of the fat body to circulating hormones.

Fig. 3.1. Quantification of native M-AKH by RP-HPLC.

a) Analysis of adult CC extract (7.8 CC pair equivalents) loaded onto a Spherisorb 5 µm C_{18} column (25 cm x 0.46 cm). Solvent A was 0.1% TFA; solvent B was 0.1% TFA in 60% acetonitrile. The gradient used is indicated by the dotted line. The flow rate was 1 ml/min.

b) Analysis of 200 ng of synthetic M-AKH. Conditions as in a).


0.1
Absorbance at 210nm
0
7.8CC
100
80
60 %B
40
20
0
24
Time(min)


0.08
Absorbance at 210nm
0
200ng,M-AKH
100
80
60 %B
40
20
0
24
Time(min)

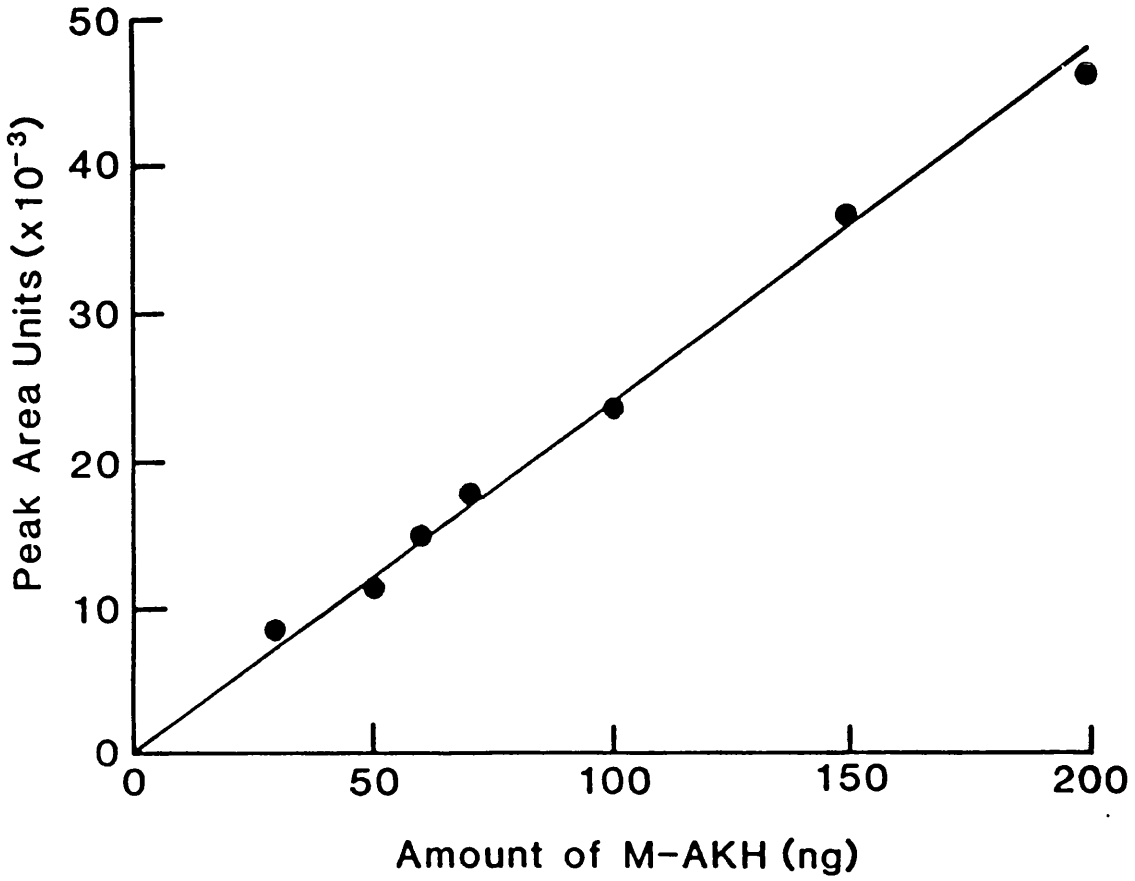


Fig. 3.2. Standard curve for the quantification of M-AKH by HPLC. Peak areas were integrated by a Gilson Model 620 Data Master during calibration analyses as illustrated in Fig. 3.1b.

Fig. 3.3. Separation of labelled [Tyr^1] M-AKH from free $Na^{125}I$. The iodination mixture was loaded onto a primed C_{18} SepPak cartridge, washed with 0.25 M phosphate buffer and eluted by a graded series of methanol solutions (3 x 600 µl each). A 10 µl aliquot from each fraction was counted in a Type N550A scintillation counter (Ecko Electronics).

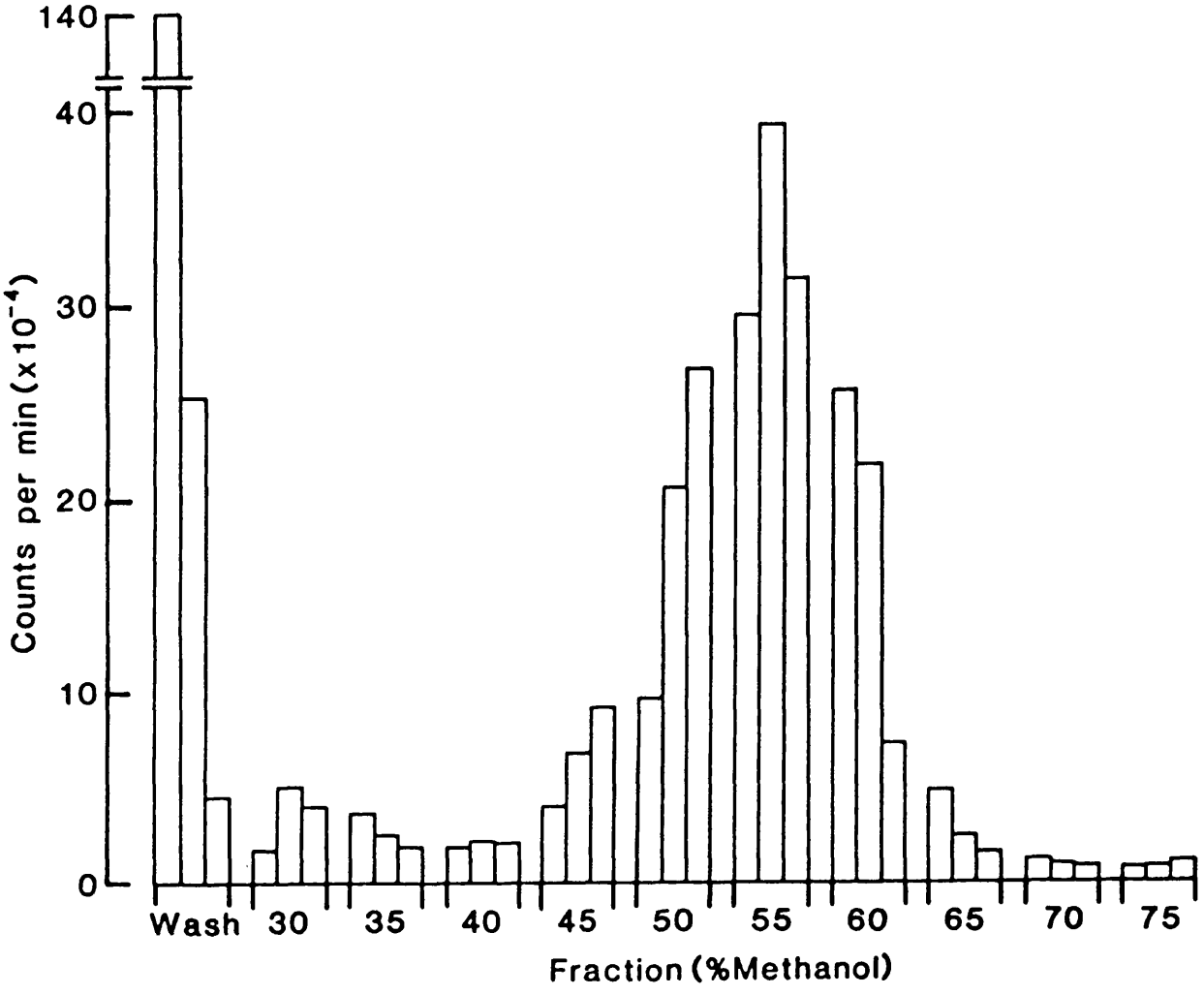
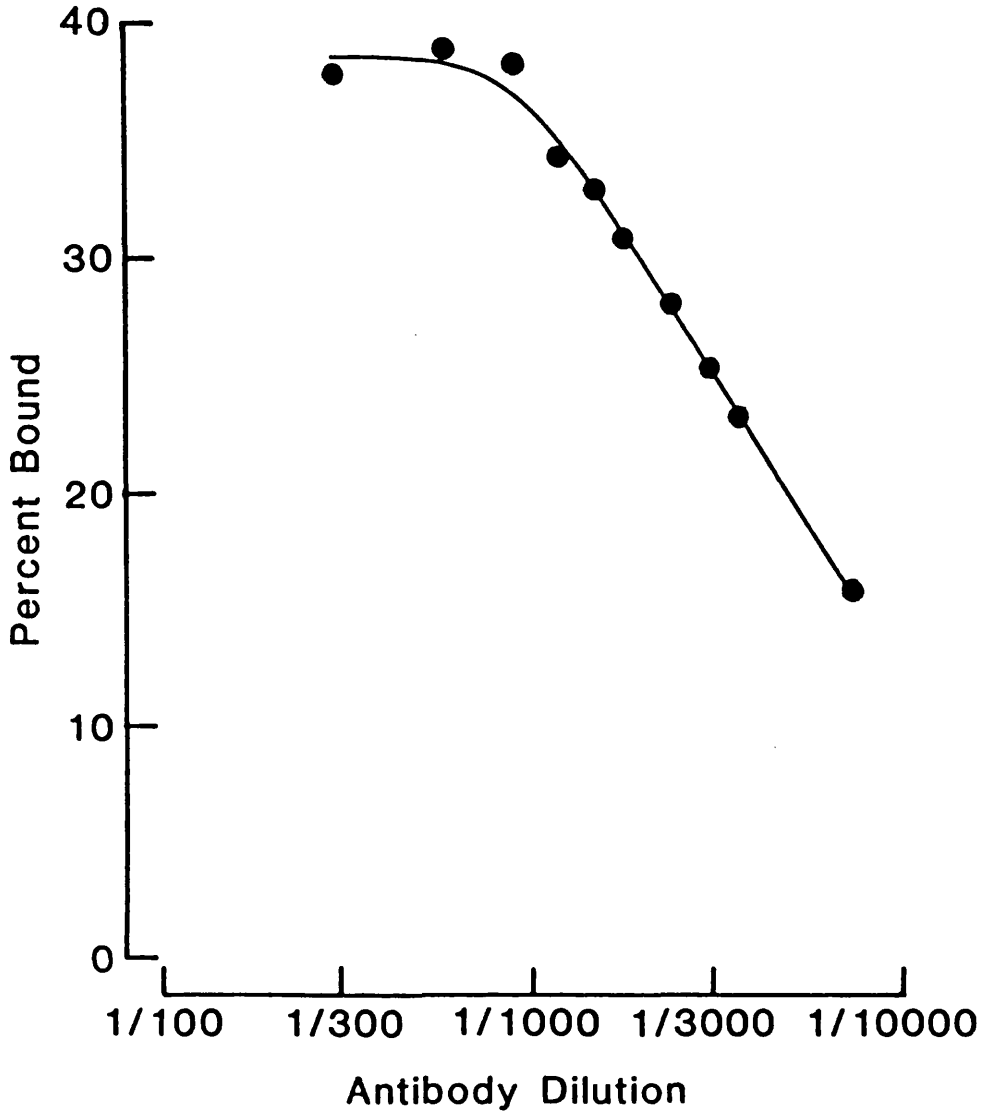

140
40
30
20
10
0
Counts per min (x10⁻⁴)
Wash
30
35
40
45
50
55
60
65
70
75
Fraction (%Methanol)

Fig. 3.4. RIA assessment of the antibody titre in serum from rabbit 158 following the terminal bleed. 50 µl of serum (1/600) was incubated overnight with 50 µl of buffer and 50 µl of labelled peptide (approximately 9,000 c.p.m.). The incubation was terminated by the addition of 500 µl of charcoal solution. The mixture was incubated at 4°C for 7-8 min and then centrifuged at 4,500 r.p.m. for 15 min. The supernatant was removed and the pellet counted in an LKB minigamma counter.


40
30
20
10
0
Percent Bound
1/100
1/300
1/1000
1/3000
1/10000
Antibody Dilution

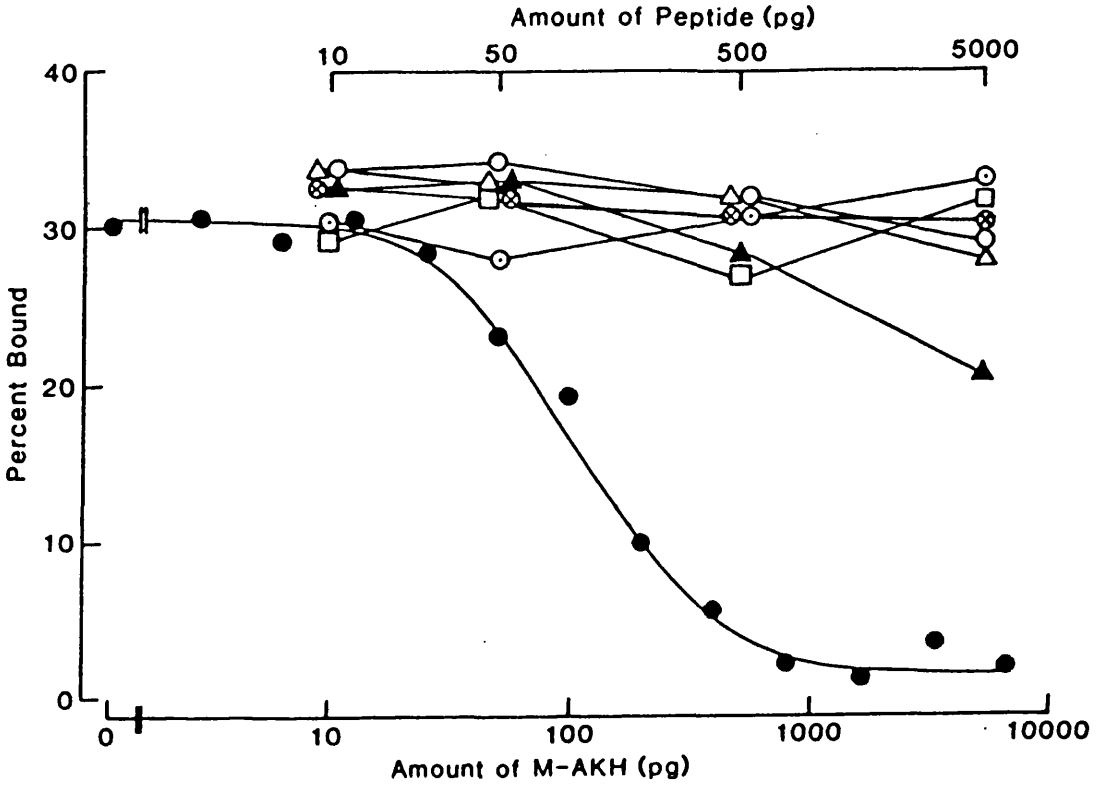


Fig. 3.5. Competition assay to determine the specificity of the RIA. Unlabelled peptides were incubated with serum (at a final concentration of 1/1800) and labelled peptide (9,000 cpm). The peptides assayed were M-AKH (●), AKH-I (⊙), AKH-II(L) (□), M-II (⊗), HTF-II (△), LK-II (▲) and LK-VI (○).

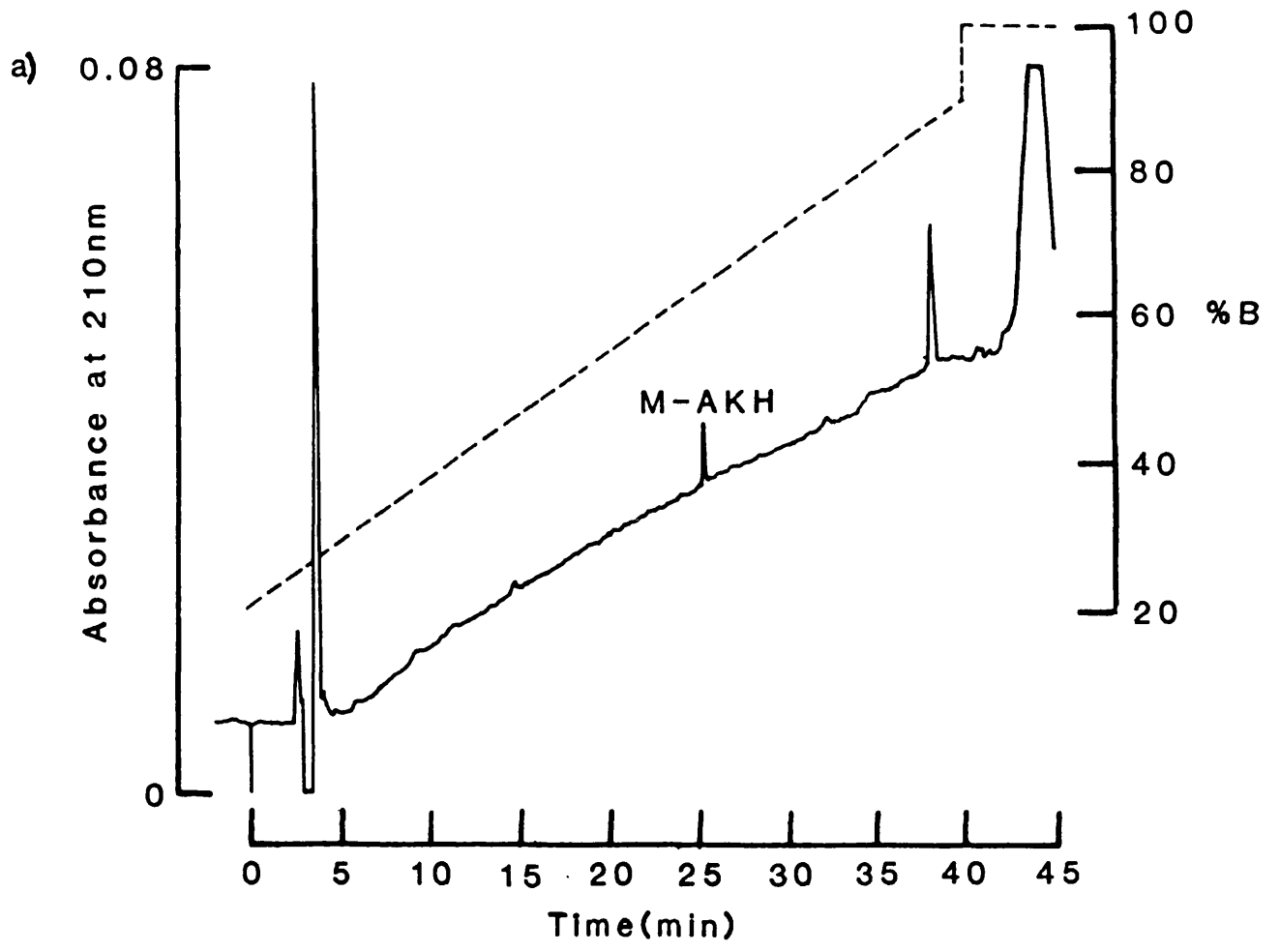
Table 3.1. Quantification of M-AKH in adult and larval tissues from Manduca by RIA. Tissues were homogenised in methanol : water : acetic acid (90:9:1) and centrifuged at 12,000 r.p.m. for 5 min. The supernatants were dried down and resuspended in 1% TFA. The samples were then lipid extracted with ethyl acetate and processed through a C_{18} SepPak cartridge. This material was dried down, resuspended in RIA buffer and assayed. Values represent mean ± standard error (SE) for n samples.

Tissue	M-AKH Content (ng/tissue)	n
Adult CC	21.00 ± 0.88	5
Adult Brain	0.67 ± 0.08	4
Adult Nerve Cord	0.08 ± 0.01	4
Larval CC	2.20 ± 0.14	4
Larval Brain	0.06 ± 0.02	5
Larval Nerve Cord	0.11 ± 0.05	5

Fig. 3.6. Identification of M-AKH immunoreactive material.

a) HPLC analysis of Manduca adult CC extract (1 pair equivalent). The chromatographic conditions were the same as those described in Fig. 3.1 except for the gradient, indicated by the dotted line. Fractions were collected by a Gilson Model 201/202 fraction collector at 1 min intervals. The peak eluting at 25.5 min is identified as M-AKH on the basis of the retention time of synthetic M-AKH.

b) RIA of aliquots from the fractions collected in a) and from HPLC of larval CC. Aliquots represented approximately 0.05 adult CC pair equivalents per tube and 0.4 larval CC pair equivalents. Each aliquot was lyophilised and then resuspended in 50 µl of RIA buffer for the assay. Aliquots outside the region of M-AKH elution were pooled for the RIA.


a)
0.08
0
Absorbance at 210nm
100
80
60
40
20
%B
M-AKH
0 5 10 15 20 25 30 35 40 45
Time(min)

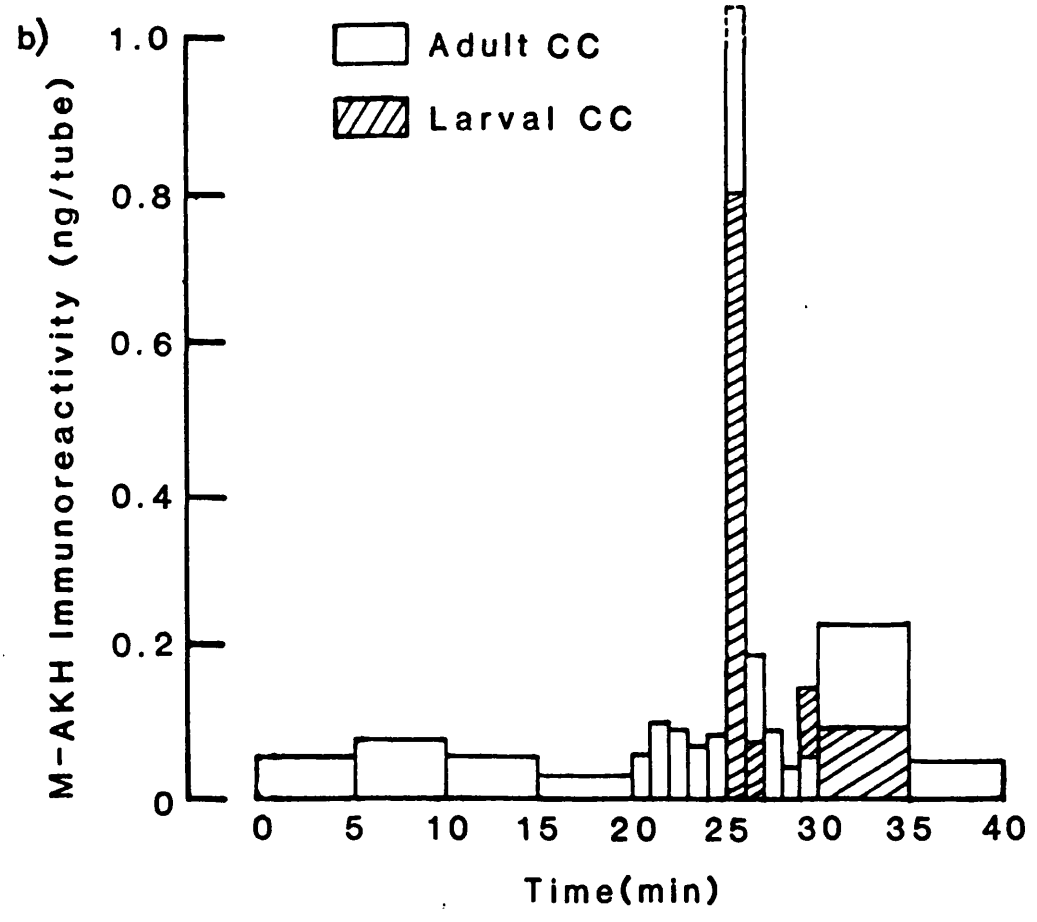
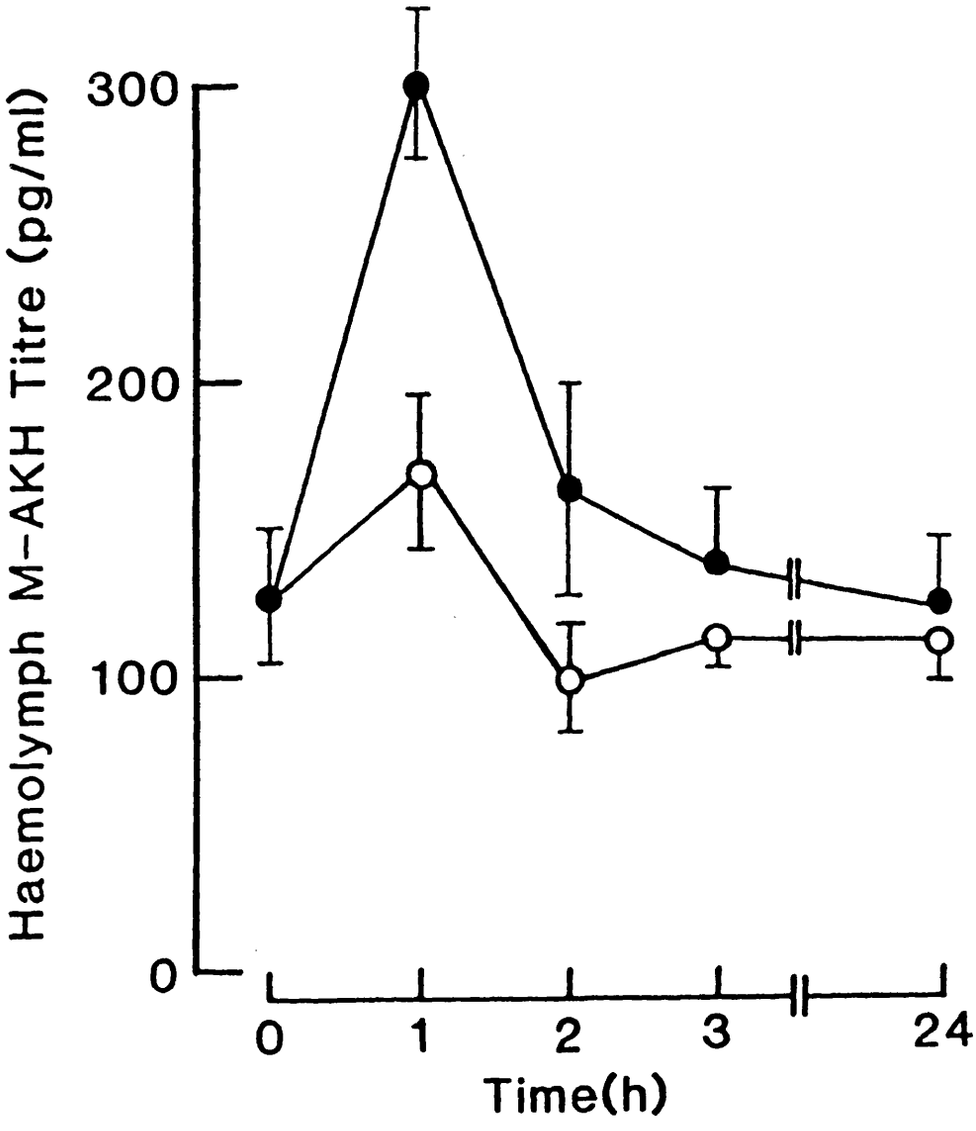

b)
Adult CC
Larval CC
1.0
0.8
0.6
0.4
0.2
0
M-AKH Immunoreactivity (ng/tube)
0 5 10 15 20 25 30 35 40
Time(min)

Fig. 3.7. Determination of M-AKH immunoreactivity in haemolymph of starved and fed larvae. Day 3, fifth instar larvae were bled at the times indicated after the start of the experiment. Haemolymph was frozen, boiled and then centrifuged at 2,000 r.p.m. for 5 min and the supernatant removed and processed through a C_{18} SepPak cartridge. The eluted material was lyophilised and resuspended in RIA buffer (50 µl) and assayed. Points and vertical bars for starved larvae (●) and fed larvae (○) represent means ± SE (n = 5 for each point).


Haemolymph M-AKH Titre (pg/ml)
300
200
100
0
0
1
2
3
24
Time(h)

CHAPTER 4. BIOLOGICAL ACTIVITIES OF M-AKH

INTRODUCTION

The corpora cardiaca (CC) of Manduca have been shown to contain a factor (or factors) which causes hyperlipaemia when injected into adult moths (Beenakkers et al., 1978) and fat body glycogen phosphorylase activation when injected into larvae (Ziegler, 1979). The latter activity is thought to be important in the regulation of carbohydrate metabolism during the period of starvation which occurs around the larval moult (Siegert and Ziegler, 1983). In adult Manduca the CC factor regulates lipid metabolism during flight, but does not appear to regulate carbohydrate metabolism during flight despite its ability to activate adult fat body phosphorylase (Ziegler and Schulz, 1986a, 1986b).

A peptide that causes hyperlipaemia in moths and phosphorylase activation in larvae when injected, has been isolated and sequenced from the CC of adult Manduca (Ziegler et al., 1984; 1985). Initially it was suggested that this peptide (M-AKH) was different to the larval glycogen phosphorylase activating hormone (GPAH) (Ziegler et al., 1986), however recent evidence from the bioassay of CC extracts has led Ziegler et al. (1987) to conclude that M-AKH is in fact identical to GPAH.

In an attempt to clarify the physiological roles of M-AKH in larval and adult Manduca, I have quantitatively assayed synthetic M-AKH for adipokinetic activity in moths and glycogen phosphorylase activation in larvae, comparing these activities with data from CC

extract bioassays. Synthetic M-AKH was also assayed for cardio-activity in larval Manduca.

MATERIALS AND METHODS

Experimental Animals

Manduca sexta were reared in a colony at the University of Bath as described in Chapter 3. Adipokinetic assays were performed with day 1 adult moths of both sexes, starved overnight before the assay to minimize the differences in the metabolic fuel stores between individual moths. Phosphorylase assays were performed with day 2, fifth instar larvae. Day 3, fifth instar larvae were used for the determination of the haemolymph titre of M-AKH and for the cardioactivity assay.

Assay Reagents

Synthetic M-AKH test solutions were prepared by aqueous dilution from a stock solution of 0.1 mg/ml in 10% acetonitrile. CC extracts were prepared, as reported in Chapter 3, by a modification of the method reported by Jaffe et al. (1986). Phosphoglucomutase and glucose 6-phosphate dehydrogenase were purchased from Boehringer-Mannheim, Lewes, U.K. All other reagents were of analytical grade and were purchased from Sigma.

Oyster glycogen (from Sigma) was treated with charcoal to remove traces of AMP. 3 g of glycogen was stirred with 2 g of activated charcoal in distilled water for 1 h. The glycogen solution was separated from the charcoal by centrifugation at 10,000 r.p.m. for 15 min in an MSE High Speed 18 centrifuge. The

supernatant (glycogen solution) was filtered through a Whatman GF-D filter and a Whatman sterile membrane filter (0.45 µm). An equal volume of 95% ethanol was added to the filtrate to precipitate the glycogen which was then recovered by filtration using Whatman No. 5 filter paper. The precipitated glycogen was washed with ethanol and ether and stored in a desiccator at 4°C.

Adipokinetic Assay

Haemolymph was collected immediately before or 100 min after injection of 10 µl of the test solution into the abdomen of the moth. A 5 µl sample of haemolymph was collected in a pyrex micropipette by puncturing the dorsal aorta in the abdominal region. The sample was transferred to 1 ml of concentrated sulphuric acid in a chloroform/methanol washed test-tube. The samples were placed in a boiling water bath for 10 min and then cooled in cold water. A 100 µl aliquot was removed from each sample, added to 1 ml of vanillin reagent (13 mM vanillin in 80% concentrated phosphoric acid), vortex mixed immediately and left to stand at room temperature for 30 min. The absorbance of each sample was then measured at 546 nm in a Philips Pye Unicam 8650 spectrophotometer. A calibration curve was prepared using standards prepared from a stock solution of cholesterol in methanol (0.8 mg/ml, since 0.8 mg of cholesterol has an absorbance equivalent to 1.0 mg of total lipid - Barnes and Blackstock, 1973).

Glycogen Phosphorylase Assay

Larvae were lightly anaesthetised with CO_2 prior to injection

with 10 µl of the test solution. Each larva was replaced on artificial diet for 10 min before the fat body was rapidly removed. The excised tissue was rinsed quickly in Manduca saline (40 mM KCl, 4 mM NaCl, 18 mM $MgCl_2$, 3 mM $CaCl_2$, 1.5 mM Na_2HPO_4, 1.5 M NaH_2PO_4, 193 mM sucrose, pH 6.5), lightly blotted on filter paper and homogenised in 1 ml of buffer (50 mM triethanolamine acetate, 5 mM Na_2EDTA, 20 mM NaF, 1 mM phenylthiourea (PTU), pH 7.0). The excision-homogenization took less than 1 min. Homogenates were stored on ice prior to centrifugation at 11,600 g for 15 min at 4°C in an MSE MicroCentaur. The infranatant was withdrawn and used for the assay.

Phosphorylase was assayed in the direction of glycogen degradation at 25°C by a coupled enzyme assay which followed the reduction of NADP (Ziegler et al., 1979). The assay mixture for the measurement of phosphorylase a contained the following components in a final volume of 1.0 ml: 42 mM triethanolamine acetate, 5.5 mM imidazole, 2 mM Na_2EDTA, 1.4 mM dithiothreitol, 80 mM KH_2PO_4, 5.1 mM magnesium acetate, 0.6 mM NADP, 0.002% BSA, 10 mg/ml glycogen, 1.24 µM glucose 1,6-diphosphate, 0.98 units of phosphoglucomutase, 0.43 units of glucose 6-phosphate dehydrogenase and 50 µl of the phosphorylase preparation at pH 7.0. The mixture was placed in a cuvette and the absorbance monitored continuously at 340 nm in a Cecil CE 373 spectrophotometer for 15 min. The change in absorbance due to the reduction of NADP was recorded with a Rikadenki chart recorder. To measure the total phosphorylase activity within the sample 50 µl of 40 mM AMP was added to the mixture and the absorbance was monitored for a further 15 min. The

gradients displayed on the chart recorder were measured by hand and used to calculate the percentage of active phosphorylase (phosphorylase a) within the sample.

Cardioactivity Assay

Cardioactivity was assayed using a semi-isolated larval heart preparation developed by Platt and Reynolds (1985). The heart was left to stabilize for a few hours while perfused with Manduca saline. A 750 pmol dose of M-AKH (50 µl of 15 µM peptide) was applied onto the preparation with a syringe and the effect on cardioactivity was monitored. The contraction of the heart was monitored by the deflection of a small hook, inserted under the heart which was connected via a thread to an almost isotonic movement transducer. The transducer's output was displayed on a Servogor 120 chart recorder. A 5 pmol dose of serotonin (50 µl of 0.1 µM 5-HT) was used to demonstrate the responsiveness of the heart to a known cardioaccelerator.

RESULTS

The activity of synthetic M-AKH was determined by its effect on haemolymph lipid levels, fat body glycogen phosphorylase activity and on the beating of the heart. Synthetic M-AKH elicited lipid mobilisation in adult moths with a maximal increase of 33 mg/ml in response to the injection of 2 pmol of peptide (Fig. 4.1). The dose required to produce a 50% maximal response may be estimated to be approximately 0.3 pmol of M-AKH.

The adipokinetic activity of adult CC extracts was also investigated (Fig. 4.2). A partial dose-response curve was obtained which indicated a maximal increase of 30 mg/ml in response to a dose of 0.1 CC equivalents and a 50% maximal response to 0.005 CC equivalents (Fig. 4.2a). The dose-response curve is shown in comparison with a theoretical dose-response curve for CC extract which was replotted from the synthetic M-AKH dose-response curve by assuming a titre of 21 pmol/CC as reported in Chapter 3. At low doses (less than 0.01 CC equivalents) the adipokinetic response to CC extract was greater than that predicted from the theoretical curve. However at doses above 0.1 CC equivalents the observed response resembled the predicted response to higher doses of CC extract. If the curves are replotted to show the response in terms of the percentage of the maximal response in each experiment (Fig. 4.2b), the curve for the CC extract appears to be displaced to the left of the curve derived from the synthetic M-AKH data. This suggests that the M-AKH content of the adult CC is insufficient to account for all of the adipokinetic activity in the CC and may therefore indicate the activity of a second adipokinetic factor from the CC.

Larval fat body glycogen phosphorylase was maximally activated (65% phosphorylase a) following the injection of a 2 pmol dose of synthetic M-AKH (Fig. 4.3). 50% maximal activation would be elicited by a dose of approximately 0.06 pmol of M-AKH. The larval phosphorylase response therefore appears to be more sensitive to M-AKH than the adult adipokinetic response.

M-AKH was not cardioactive when assayed on a semi-isolated larval heart (Fig. 4.4) No change was observed in the amplitude or rate of the larval heartbeat when a very large dose (750 pmol) of M-AKH was applied. The responsiveness of the preparation was demonstrated by the application of serotonin (5 pmol) which stimulated both the rate and amplitude of contraction as expected.

DISCUSSION

The discovery of an adipokinetic factor in the CC of Manduca which appeared to be chemically different to locust AKH, supported the hypothesis that there was more than one adipokinetic hormone in insects (Beenakkers et al., 1978). Subsequently a number of AKH-like peptides were isolated from a variety of insect species. Among these were two additional peptides from locusts (Siegert et al., 1985) and two peptides from the cockroach Periplaneta americana (Witten et al., 1984). Recently, two AKH/RPCH family peptides have been sequenced from the grasshopper Romalea microptera (Gäde et al., 1988). The reason for the existence of two peptides with overlapping biological activities in the same species is unknown.

Ziegler and Gäde (1984) were unable to separate the glycogen phosphorylase activating hormone (GPAH) from the adipokinetic hormone (M-AKH) in adult Manduca CC by conventional chromatographic techniques. Subsequently, Ziegler et al. (1986) separated two peptides from larval CC by RP-HPLC. They concluded that M-AKH and GPAH were different peptides with overlapping biological activities. However amino acid analysis and bioassay data have

since been reported to demonstrate the identity of both hormones with the nonapeptide M-AKH (Ziegler *et al.*, 1987).

In this study synthetic M-AKH was found to possess adipokinetic and phosphorylase activating activity in adult and larval *Manduca*, respectively. The ability of CC extract or synthetic M-AKH to activate adult fat body glycogen phosphorylase was not tested. The most economical hypothesis would seem to be that M-AKH regulates both processes *in vivo*. However a comparison of the dose-response curve for adipokinetic activity in response to adult CC extracts with a theoretical dose-response curve suggests that M-AKH may not be the only adipokinetic factor within the adult CC. The maximum response to injected CC extract appeared to be the same as that elicited by M-AKH, but at lower doses the activity of the CC extract was not fully accounted for by the M-AKH content alone.

A related insect, the corn earworm moth, *Heliothis zea* possesses two AKH/RPCH family peptides. One of these (H-AKH-I) is identical to M-AKH, whilst a second peptide (H-AKH-II) with less adipokinetic activity, has recently been sequenced from *Heliothis* CC (pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-AsnNH_2, Jaffe *et al.*, 1988b). This discovery would appear to strengthen the hypothesis that there is a second peptide in *Manduca* CC, but the isolation of such a peptide remains a task for the future.

RIA analysis of CC extracts following HPLC separation indicated a second peak of immunoreactive material distinct from that due to M-AKH (see Chapter 3). Although it was not possible to obtain a sample of the second *Heliothis* AKH/RPCH peptide for comparison, it

would seem unlikely that the M-AKH immunoreactive material eluting later than M-AKH is the same peptide. In the HPLC system used by Jaffe et al. (1988b), the second Heliothis peptide eluted at a lower concentration of acetonitrile than M-AKH, unlike the M-AKH immunoreactive material reported here. In any case, on the basis of its C-terminal sequence the Heliothis peptide would not be expected to show a significant degree of cross-reactivity in the M-AKH RIA (see Chapter 3). The adipokinetic activity of the late-eluting M-AKH immunoreactive material has not yet been determined so it cannot be concluded that it is responsible for the additional bioactivity in adult CC extracts.

The unknown immunoreactive material was also present in larval Manduca CC extracts yet the adipokinetic response to larval CC extracts reported by Ziegler et al. (1987) was consistent with the identification of M-AKH as the only adipokinetic factor within larval CC. This again suggests that the putative second AKH/RPCH peptide in adult Manduca CC is probably not the same as the immunoreactive material reported in Chapter 3.

A comparison of the glycogen phosphorylase activation dose-response curve for synthetic M-AKH with the previously reported activities of larval and adult CC extracts (Ziegler et al., 1988; Ziegler, 1979) supports the hypothesis that M-AKH is identical to GPAH. The reported response of larval fat body glycogen phosphorylase to injected CC extracts (a 50% maximal response to 0.003 adult CC equivalents or 0.03 larval CC equivalents) was very consistent with the activity of synthetic M-AKH reported here (a 50% maximal response to 0.06 pmol of M-AKH) and the CC titres

in Chapter 3.

Starvation is known to cause the release of GPAH from the larval CC leading to fat body phosphorylase activation (Siegert and Ziegler, 1983). Using an M-AKH specific RIA I have shown that M-AKH is released into the haemolymph within the first hour of starvation (see Chapter 3). On this basis the phosphorylase response would also be expected to peak at about 1 h since phosphorylase activation is known to be rapid (Ziegler, 1979), however Siegert (1987) reported a peak of phosphorylase activity between 2 h and 3 h after the onset of starvation. The discrepancy between the data presented in Chapter 3 and those of Siegert (1987) may be due to differences in the laboratory cultures of Manduca used, particularly in relation to the quality of the artificial diet. Further work is required to confirm the identity of the immunoreactive material in larval haemolymph and to obtain a direct comparison of the haemolymph M-AKH titre and phosphorylase activity in the same insect. Nevertheless, the bioassay and RIA data support the hypothesis that M-AKH and GPAH are identical.

AKH/RPCH peptides can also affect the activity of muscles and the two cockroach peptides M-I and M-II were originally isolated on the basis of their cardioactivity in the cockroach (Scarborough et al., 1984) and their myotropic activity in locust leg muscle (O'Shea et al., 1984). Therefore it seemed appropriate to test synthetic M-AKH for myotropic activity in Manduca. The assay used was the semi-isolated larval heart described by Platt and Reynolds (1985). M-AKH failed to affect either the rate or amplitude of the heartbeat, even when applied in large amounts. Platt and Reynolds

(1986) had previously observed that AKH-I also lacked cardio-activity in Manduca. While this assay indicates that M-AKH is not involved in the control of the heartbeat in Manduca, it remains possible that M-AKH may affect other muscles.

AKH-I has been shown to inhibit protein synthesis in the locust fat body (Carlisle and Loughton, 1986). The effect of synthetic M-AKH and CC extracts on protein synthesis in Manduca fat body has been investigated in our laboratory. Neither synthetic M-AKH (at a dose of 20 nM in vitro or 10-50 nM in vivo) nor CC extract (1 CC equivalent per fat body incubation or per insect) caused a significant change in fat body protein synthesis (Prosser, 1987). The relevance of this finding remains unclear as the physiological explanation for the effect of AKH-I on protein synthesis in locusts is not known (Orchard, 1987).

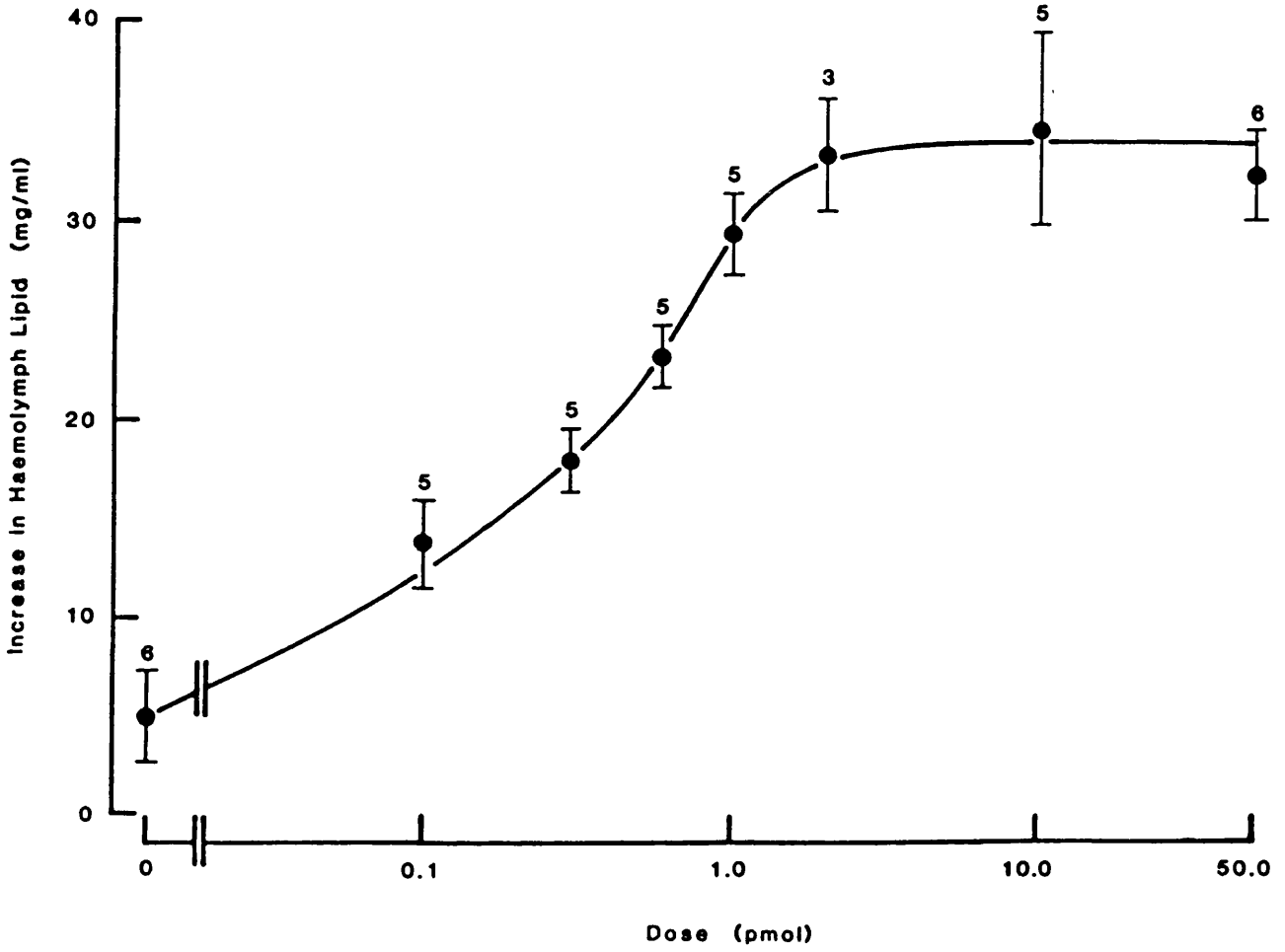
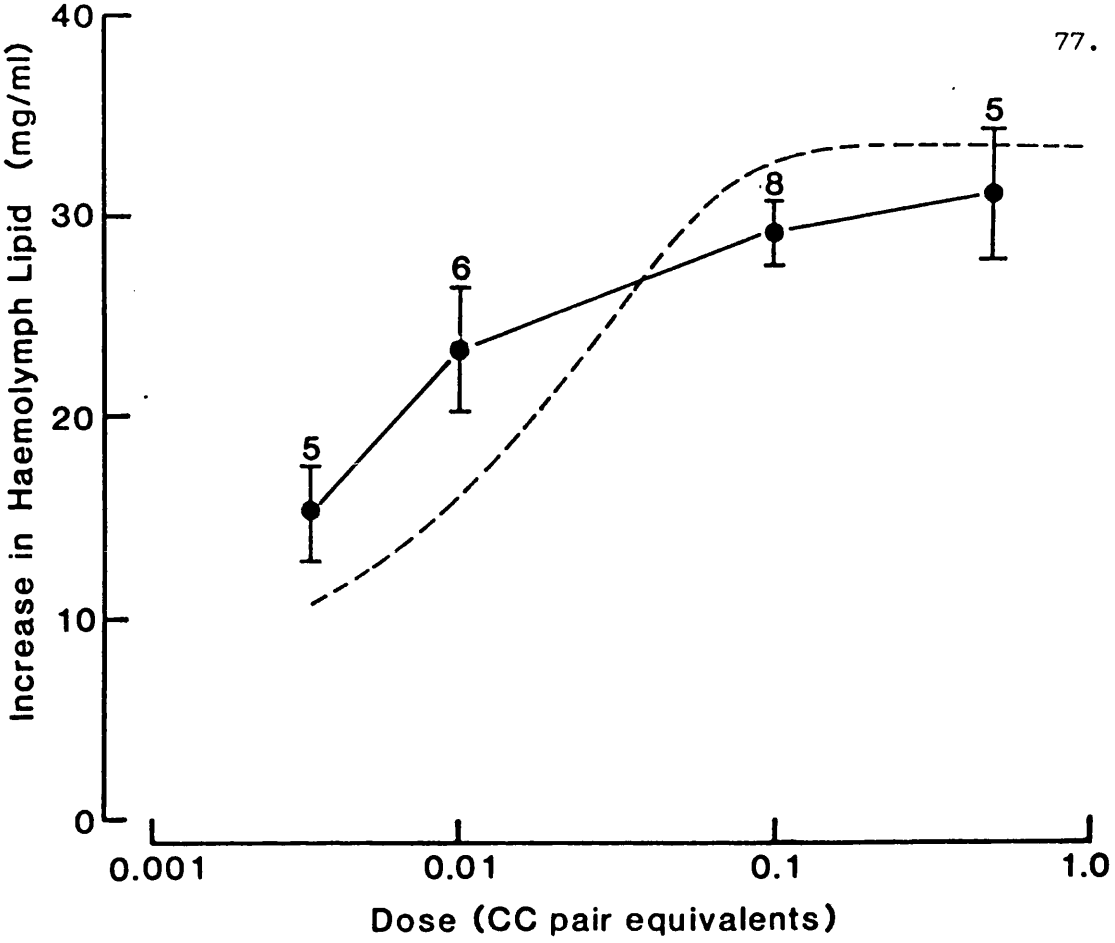


Fig. 4.1. Adipokinetic assay of synthetic M-AKH in adult Manduca. Haemolymph samples (5 µl) were collected immediately before and 100 min after injection of the peptide. Haemolymph lipid levels were determined by a sulphophosphovanillin method. Points and vertical bars represent the mean ± S.E. for the number of samples indicated above each point.

Fig. 4.2. Adipokinetic assay of adult CC extract. Method as described in Fig. 4.1.

a) Absolute increase in haemolymph lipids is shown in comparison with the theoretical curve (dotted line) derived from the dose-response curve in Fig. 4.1 and the M-AKH content of adult CC determined by RIA (21 pmol/CC). Points and vertical bars for the observed response represent the means ± S.E. for the number of samples indicated above each point.

b) Data from a) expressed as a relative response compared with the maximum response (theoretical and observed).


Increase in Haemolymph Lipid (mg/ml)
40
30
20
10
0
5
6
8
5
0.001
0.01
0.1
1.0
Dose (CC pair equivalents)

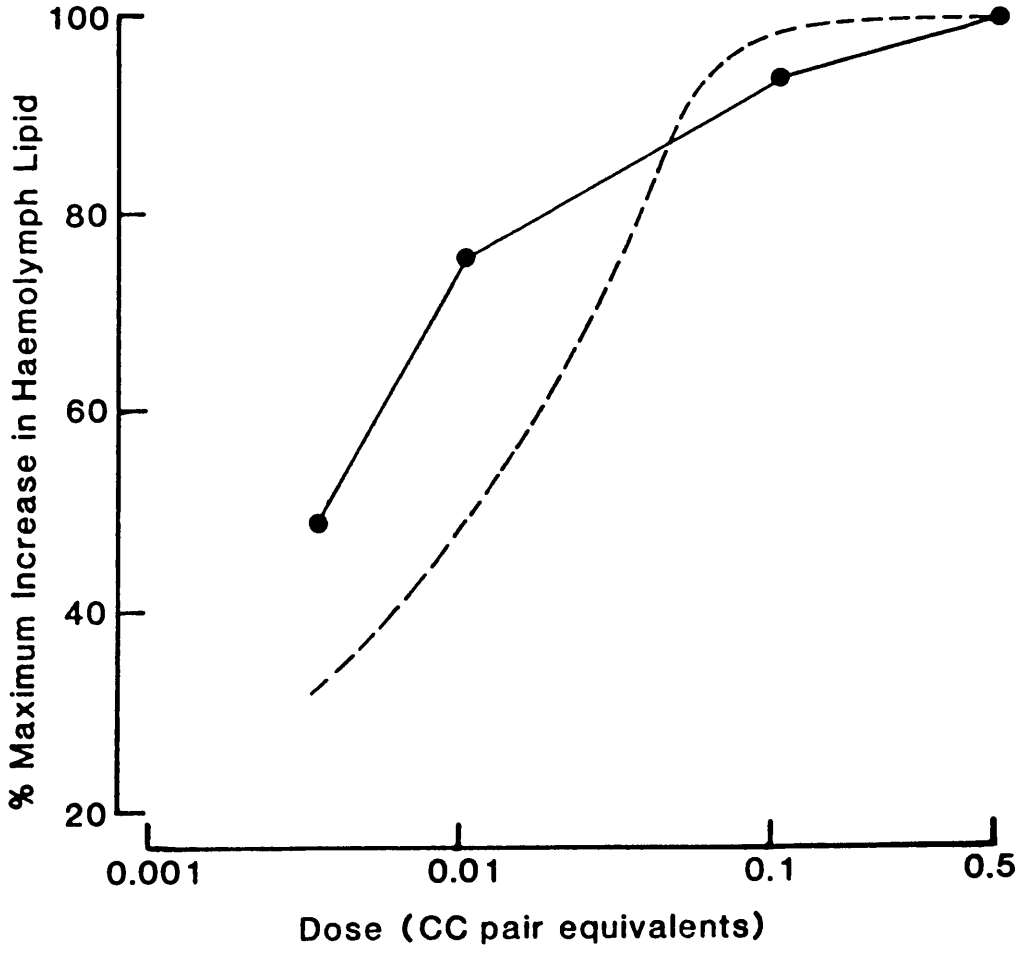
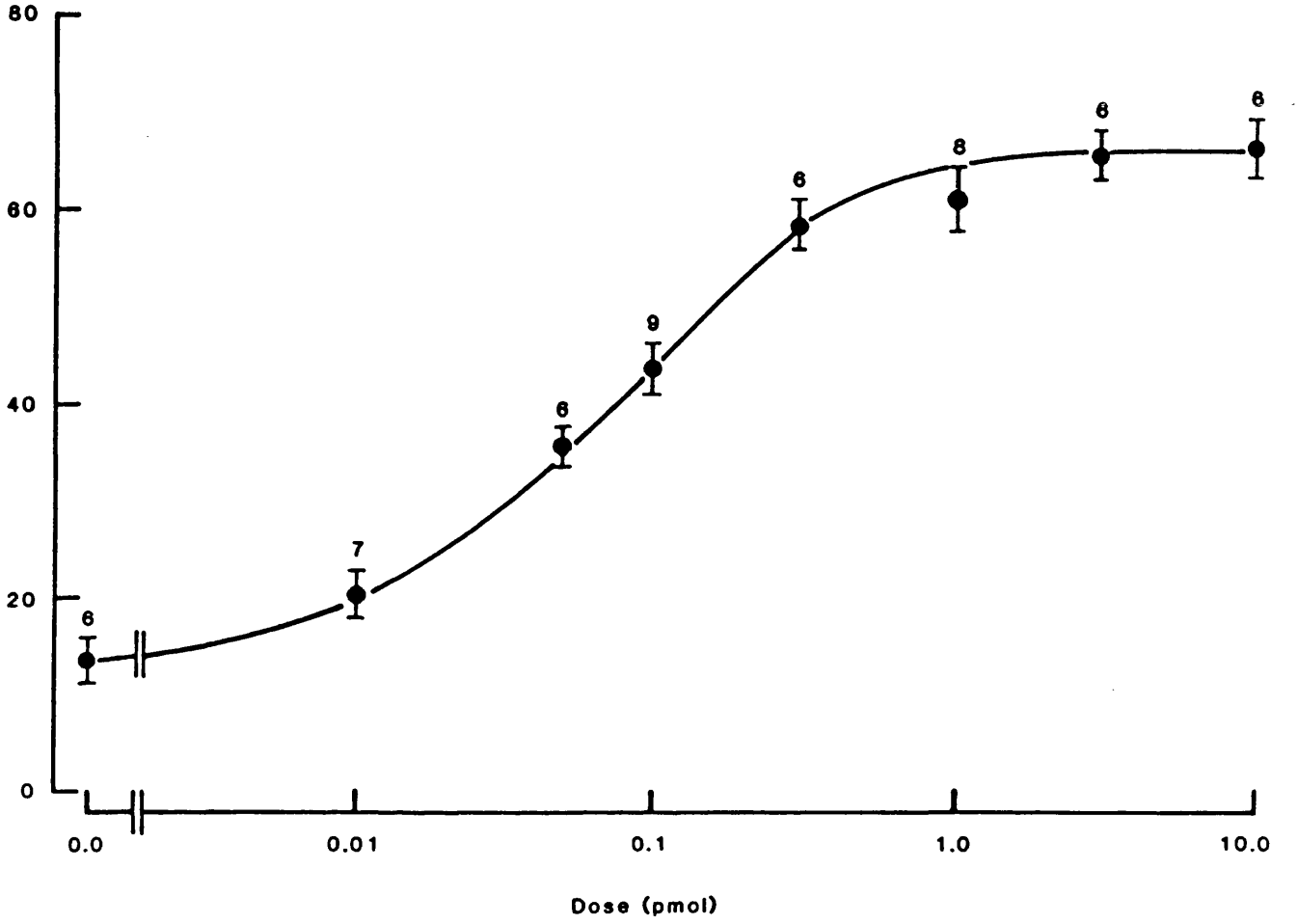

% Maximum Increase in Haemolymph Lipid
100
80
60
40
20
0.001
0.01
0.1
0.5
Dose (CC pair equivalents)

Fig. 4.3. Fat body glycogen phosphorylase assay of synthetic M-AKH. Day 2, fifth instar larvae were injected with the test dose of peptide. After 10 min the fat body was rapidly removed and homogenised in buffer (50 mM TEA, 5 mM Na_2EDTA, 20 mM NaF, 1 mM PTU, pH 7.0) centrifuged at 11,000 g for 15 min and the infranatant withdrawn for the assay of glycogen phosphorylase in the direction of glycogen degradation as described by Ziegler et al. (1979). Points and vertical bars represent means ± S.E. for the number of samples indicated above each point.


80
60
40
20
0
6
7
6
9
6
8
6
6
0.0
0.01
0.1
1.0
10.0
Dose (pmol)

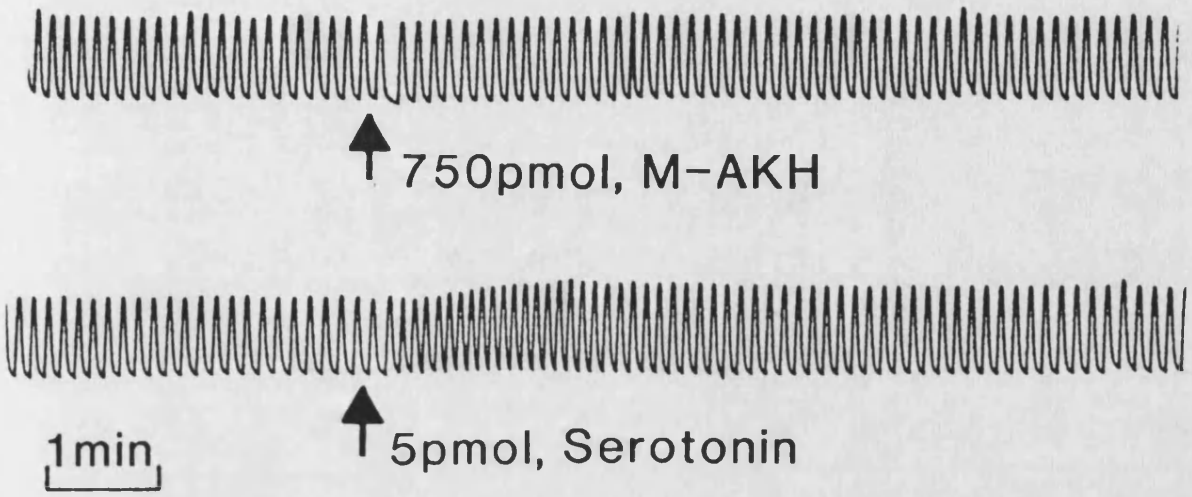


Fig. 4.4. Cardioactivity of synthetic M-AKH (top trace) compared with serotonin (bottom trace) assayed on a semi-isolated larval Manduca heart at the doses indicated.

CHAPTER 5. PHARMACOLOGY OF THE ADIPOKINETIC RESPONSE TO M-AKH

INTRODUCTION

The characteristics of a peptide receptor may be investigated by assaying synthetic or natural peptides which are structurally related to the native peptide. Shortly after the sequence of locust AKH-I was determined (Stone *et al.*, 1976), the structure-activity relationships of a number of synthetic analogues of varied length and sequence were investigated in the locust (Stone *et al.*, 1978).

The discovery of a family of insect peptides related to locust AKH-I and the prawn red pigment concentrating hormone (RPCH) has provided an additional impetus for the study of structure-activity relationships. Naturally occurring AKH/RPCH family peptides have been assayed for adipokinetic activity in the locust (Goldsworthy *et al.*, 1986a, 1986b) and hypertrehalosaemic activity in the cockroach (Gäde, 1986). These studies have provided information on the amino acid residues and sequences that are required for biological activity in the recipient insects (Goldsworthy and Wheeler, 1986).

I have investigated the adipokinetic activity of AKH/RPCH family peptides in adult *Manduca*. Synthetic AKH/RPCH peptides were assayed separately and in competition with the native peptide, M-AKH. The results will be discussed in relation to the characteristics of the M-AKH receptor of *Manduca* fat body.

MATERIALS AND METHODS

Sources of Peptides

M-AKH was synthesised as described in Chapter 2. Locust AKH-I was purchased from Peninsula Laboratories, St. Helens, U.K. The other synthetic peptides were gifts from the following sources: Prawn red-pigment concentrating hormone (RPCH) from Professor G.J. Goldsworthy, Birkbeck College, London, U.K.; Locust adipokinetic hormones II (AKH-II(S) and AKH-II(L)) from Professor W. Mordue, University of Aberdeen, U.K.; Cockroach myoactive factors (M-I and M-II) from Professor M. O'Shea, University of Geneva, Switzerland; Carausius hypertrehalosaemic factor II (HTF-II), Nauphoeta hypertrehalosaemic hormone (HTH) and the Romalea peptides (RoI and RoII) from Dr. G. Gäde, University of Dusseldorf, F.R.G. Stock solutions were quantified by HPLC.

Adipokinetic Assay

A 50 pmol dose of each peptide was assayed for adipokinetic activity by the sulphophosphovanillin method described in Chapter 4. Those peptides which displayed significant activity at this dose were assayed at lower doses to obtain a dose-response curve for each peptide.

For the competition assays test solutions were prepared so that 15 µl of solution contained 2 pmol of M-AKH and 20 pmol of another AKH/RPCH peptide. This solution was assayed for adipokinetic activity as described previously.

RESULTS

Apart from M-AKH itself, only five of the peptides tested at a supramaximal dose of 50 pmol stimulated a significant increase in haemolymph lipid levels (Table 5.1). If the response to M-AKH is taken as defining the maximal adipokinetic response then only M-AKH is a full agonist. Three other peptides were found to be partial agonists. HTF-II from the stick insect, Carausius morosus caused a 50% maximal response at approximately 0.3 pmol as did the native peptide, however the maximum increase in lipids following HTF-II injection was only 22.5 mg/ml (68% of the maximal response to M-AKH, Fig. 5.1). Locust AKH-I stimulated a maximum increase of 21 mg/ml (64% of the maximal response to M-AKH). In this case a 50% maximal response was elicited by 2-3 pmol of peptide (Fig. 5.2). The myoactive and hypertrehalosaemic peptide M-II from the cockroach Periplaneta americana elicited a maximum increase of 18 mg/ml (55% of the maximal response to M-AKH) and the dose required for a 50% maximal response was approximately 10 pmol (Fig. 5.3). HTH from the cockroach Nauphoeta cinerea and RPCH from prawns both elicited a small but significant increase in haemolymph lipids at a dose of 50 pmol (8.8 and 9.0 mg/ml, respectively) however the responses to 10 pmol of HTH (7.1 ± 2.4 mg/ml, n = 8) and to 20 pmol of RPCH (5.7 ± 2.3 mg/ml, n = 6) were not significantly different from the control. All the other peptides tested failed to elevate haemolymph lipid levels significantly even at a dose of 50 pmol per insect.

A further series of assays investigated the ability of AKH/RPCH peptides to interact with M-AKH when injected into adult moths

(Table 5.2). The only peptides to produce a significant change in the adipokinetic response when co-injected in ten-fold excess with M-AKH were AKH-I and HTF-II. In both cases the response to the peptide co-injection (24.7 mg/ml for AKH-I and 22.0 mg/ml for HTF-II) was significantly lower than the response to M-AKH alone (32.3 mg/ml). In fact the co-injection responses were similar to those elicited by AKH-I and HTF-II when injected alone (22.6 mg/ml and 20.8 mg/ml respectively).

DISCUSSION

Studies on the pharmacology of the adipokinetic response in Locusta indicate that several members of the AKH/RPCH family can stimulate the maximal response although higher doses of these peptides are always required than is the case for AKH-I (Goldsworthy et al., 1986a, 1986b). Next to AKH-I, the most effective agonist in Locusta was the stick insect peptide HTF-II which elicited a maximal response at a dose of 8 pmol, compared with 2 pmol of AKH-I. The cockroach peptides M-I and M-II and the prawn peptide RPCH had virtually identical activity, stimulating the maximal response at a dose of approximately 20 pmol. HPLC-purified AKH-II(S) and AKH-II(L) failed to elicit a maximal response in Locusta even at high doses, however recent studies suggest that this may have been due to losses of peptide during the extraction procedure. Synthetic AKH-II(S) elicited a maximal response in Locusta at a dose of approximately 5 pmol but the maximum response to synthetic AKH-II(L) was only 50% of the maximal response (Goldsworthy and Wheeler, 1986). M-AKH also elicited a

truncated response in Locusta (45% of the maximal response, Wheeler et al., 1988).

The results from these studies and the earlier work on synthetic AKH analogues (Stone et al., 1978; Hardy and Sheppard, 1983) have been interpreted in terms of the sequence requirements of the locust AKH receptor. Stone et al. (1978) found that a minimum of eight amino acid residues was required for adipokinetic activity and that the N-terminal L-pyroglutamate residue was essential for full activity. The importance of the C-terminal sequence was indicated by the low activity of [Thr^{9}, $GlyNH_2^{10}$]AKH-I in which the residues at positions 9 and 10 in AKH-I were exchanged.

Using a predictive model for the secondary structure of proteins Mordue and Stone (1978) proposed that AKH-I might possess a β-turn at residues 6 to 8. Hardy and Sheppard (1983) demonstrated a marked reduction in the activity of [3,4-DehydroPro^{6}] AKH-I compared with AKH-I indicating the importance of the [Pro^{6}] residue. The studies of Goldsworthy et al. (1986a, 1986b) suggested that [Pro^{6}]-containing AKH/RPCH peptides could act as full agonists whereas the peptides which lack [Pro^{6}] always gave a truncated response. This was thought to indicate differences in the secondary structure of the two groups of peptides. Recent studies using circular dichroism spectroscopy have investigated the probability of a β-turn being present in AKH-I and related peptides (G.J. Goldsworthy, personal communication). None of the AKH/RPCH peptides appear to possess a β-turn in aqueous solutions however in the presence of SDS-micelles (which are thought to mimic the conditions

which prevail in the environment of the receptor), all the [Pro^6]-containing peptides appear to form a β-turn whereas the non-[Pro^6] containing peptides (including M-AKH and AKH-II(S)) do not. The activity of synthetic AKH-II(S) in Locusta cannot be explained in terms of a β-turn but it is thought that AKH-I forms a type I β-turn when bound to the locust fat body receptor. Gäde (1986) concluded that [Pro^6] is also important to hypertrehalosaemia in the cockroach Periplaneta americana possibly indicating a requirement for a β-turn by the cockroach fat body receptor.

The study in Manduca reported here did not investigate the requirement for an N-terminal pyroglutamate residue, however the inactivity of [Tyr^1] M-AKH reported in Chapter 2 indicates that this residue is probably essential. The importance of other residues within the sequence of M-AKH may be inferred by comparison with other AKH/RPCH peptides. The pharmacology of the adipokinetic response in Manduca may be quite different to that of Locusta as the native peptide, M-AKH, does not possess a proline residue and is not thought to adopt a β-turn structure.

M-AKH is unique among the known AKH/RPCH peptides in possessing a serine residue at position 6. This may be essential for full adipokinetic activity as none of the other peptides tested elicited the maximal response even at high doses (up to 50 pmol per insect). AKH-I and HTF-II both stimulated a 65-70% maximal response, though HTF-II was notably more active than AKH-I at low doses. The only sequence difference between these two peptides is the replacement of [Asn^3] in AKH-I by [Thr^3] in HTF-II, the latter peptide thus possessing considerable homology with M-AKH at the N-terminal

region. M-II is identical to HTF-II apart from the absence of residues 9 and 10 at the C-terminal and shows a maximal response of 55% of the full adipokinetic response. The dose response curve for M-II is quite different to that for HTF-II, the latter peptide causing an adipokinetic response at doses some fifty times less than in the case of M-II. This is presumably due to the absence of the [Gly^9] and [Thr^{10}] residues in M-II. M-AKH has only 9 residues so it would seem that [Gly^9] is important for adipokinetic activity while [Thr^{10}] is not. The Nauphoeta peptide, HTH, and RPCH (only active at a dose of 50 pmol) clearly possess very poor adipokinetic activity in Manduca, being at least 500 times less potent than M-AKH. The inactivity of the Romalea peptide RoI which differs from AKH-I by possessing a [Val^2] residue rather than [Leu^2] indicates a requirement for leucine rather than valine at position 2.

A preliminary assessment of the sequence requirements of the Manduca receptor for M-AKH therefore indicates an absolute requirement for [$pGlu^1$] and [Leu^2] for activity. [Thr^3] appears to be important for activity at low doses and the importance of [Phe^4] is suggested by the relative inactivity of the analogue [Iodo-Phe^4]M-AKH reported in Chapter 2. All the active peptides possess a [Thr^5] residue however my results do not demonstrate activity specifically due to the presence of [Thr^5] in a peptide since no peptide differed from M-AKH at this residue alone. Interestingly, the cockroach peptide leucokinin II, which has the same C-terminal tetrapeptide sequence as M-AKH, did not show significant adipokinetic activity when assayed at a dose of 50 pmol (increase in haemolymph lipid = 4.9 ± 1.3 mg/ml, n = 5). This

confirms the importance of the N-terminal sequence. The serine residues at positions 6 and 7 are unique to M-AKH and may be vital for full adipokinetic activity. All the known members of the AKH/RPCH family possess a tryptophan residue at position 8 and its conservation is assumed to indicate its requirement for activity. A comparison of the dose response curves for HTF-II and M-II suggests that the glycine amide at position 9 (the C-terminal) may be required for activity at low doses. The partial agonists at the M-AKH receptor possess a [Pro^6] residue and probably form a β-turn but it is thought to be unlikely that M-AKH forms such a turn. The present results do not permit any further comment on the secondary structure of M-AKH at the fat body receptor.

Both cockroaches and locusts have been shown to possess two AKH/RPCH peptides with overlapping biological activities. It is not known whether these peptides are active at the same or separate receptors. In the locust preliminary evidence suggested that two receptors may be present. Locusts were injected with a supramaximal dose of AKH-II, assumed to be sufficient to saturate the appropriate receptors and then a dose of AKH-I was injected. An increase in the adipokinetic response following the injection of AKH-I was observed and interpreted as evidence for a separate population of AKH-I receptors which were available for binding despite the presence of AKH-II in excess (G.J. Goldsworthy, personal communication).

In an attempt to investigate the possibility that more than one AKH receptor may be present in Manduca, a series of competition assays were performed with a 2 pmol dose of M-AKH (sufficient to

elicit the maximal response), co-injected with a 20 pmol dose of another peptide. In most cases there was no evidence of any competition and the full response was observed, however coinjection of HTF-II or AKH-I resulted in a significant reduction of the adipokinetic response. This antagonism is most economically explained as being due to competitive binding by the excess peptide at a single M-AKH receptor resulting in a response which more closely resembles that elicited by 20 pmol of HTF-II or AKH-I alone rather than the response to 2 pmol of M-AKH.

Since the majority of the peptides tested showed no ability to mobilize lipids when injected alone, it is not surprising that they showed no ability to antagonise the activity of M-AKH. However the cockroach peptide M-II which shows appreciable activity on its own, did not appear to affect the activity of M-AKH when co-injected with the native peptide. The affinity of the AKH-receptor for M-II is some fifty-fold less than for HTF-II so it may be that the competing concentration of M-II was insufficient to demonstrate any antagonistic effect. Unfortunately it was not possible to perform competition experiments with larger doses of M-II. The failure of M-II to give an additive effect with M-AKH suggests that like HTF-II and AKH-I it does not interact with a second receptor type but with the same receptor that accepts M-AKH itself.

In this model, the failure of the three partial agonists to produce a full adipokinetic response is due to their failure to interact with the receptor in qualitatively the same way as does M-AKH. Further studies are required to explore this model more fully, for example the use of synthetic M-AKH analogues that

closely resemble M-AKH, especially at positions 6 and 7, would be particularly helpful.

Table 5.1. Adipokinetic activity of AKH/RPCH peptides in adult Manduca

Peptide	Sequence	Maximum Response
M-AKH	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-GlyNH_2	32.4 ± 2.2**
HTF-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	20.5 ± 1.8**
M-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-TrpNH_2	18.0 ± 1.3**
AKH-I	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	20.7 ± 1.7**
RoI	pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	4.7 ± 1.2
HTH	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-ThrNH_2	8.8 ± 1.8*
M-I	pGlu-Val-Asn-Phe-Ser-Pro-Asn-TrpNH_2	2.5 ± 1.1
RoII	pGlu-Val-Asn-Phe-Ser-Thr-Gly-TrpNH_2	4.0 ± 2.2
AKH-II(S)	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-TrpNH_2	1.2 ± 1.1
AKH-II(L)	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-TrpNH_2	2.5 ± 2.3
RPCH	pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH_2	9.0 ± 1.1*

Maximum response in mg/ml to 50 pmol of peptide. Values represent mean ± S.E. (where $n > 6$). Asterisks indicate which responses were significantly different from the control according to a t-test (**$p > 0.01$; *$p > 0.05$).

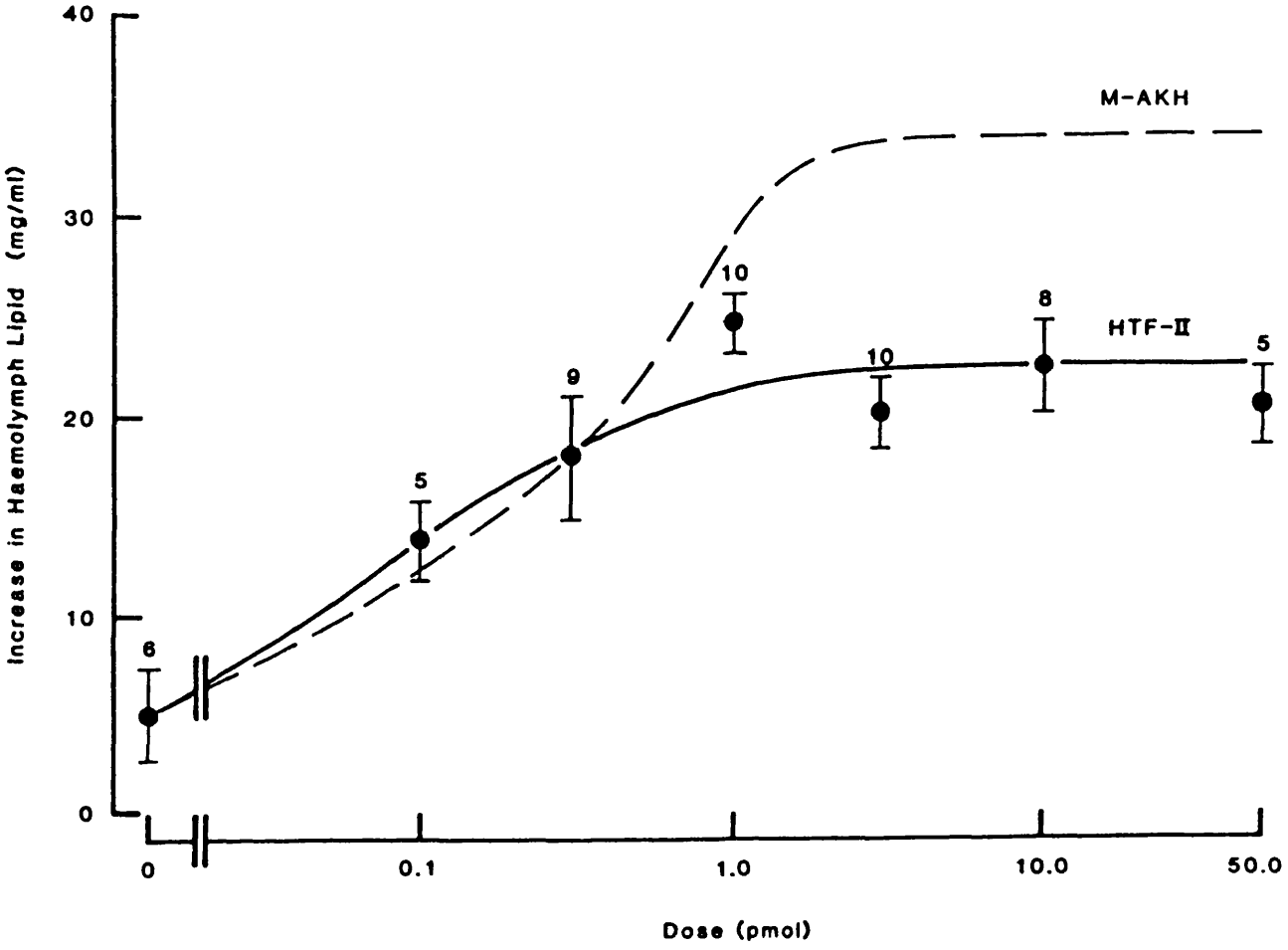


Fig. 5.1. Adipokinetic assay of synthetic HTF-II in adult Manduca. Dotted line indicates the dose-response curve for M-AKH. Points and bars represent means ± S.E. for the number of samples indicated above each point.

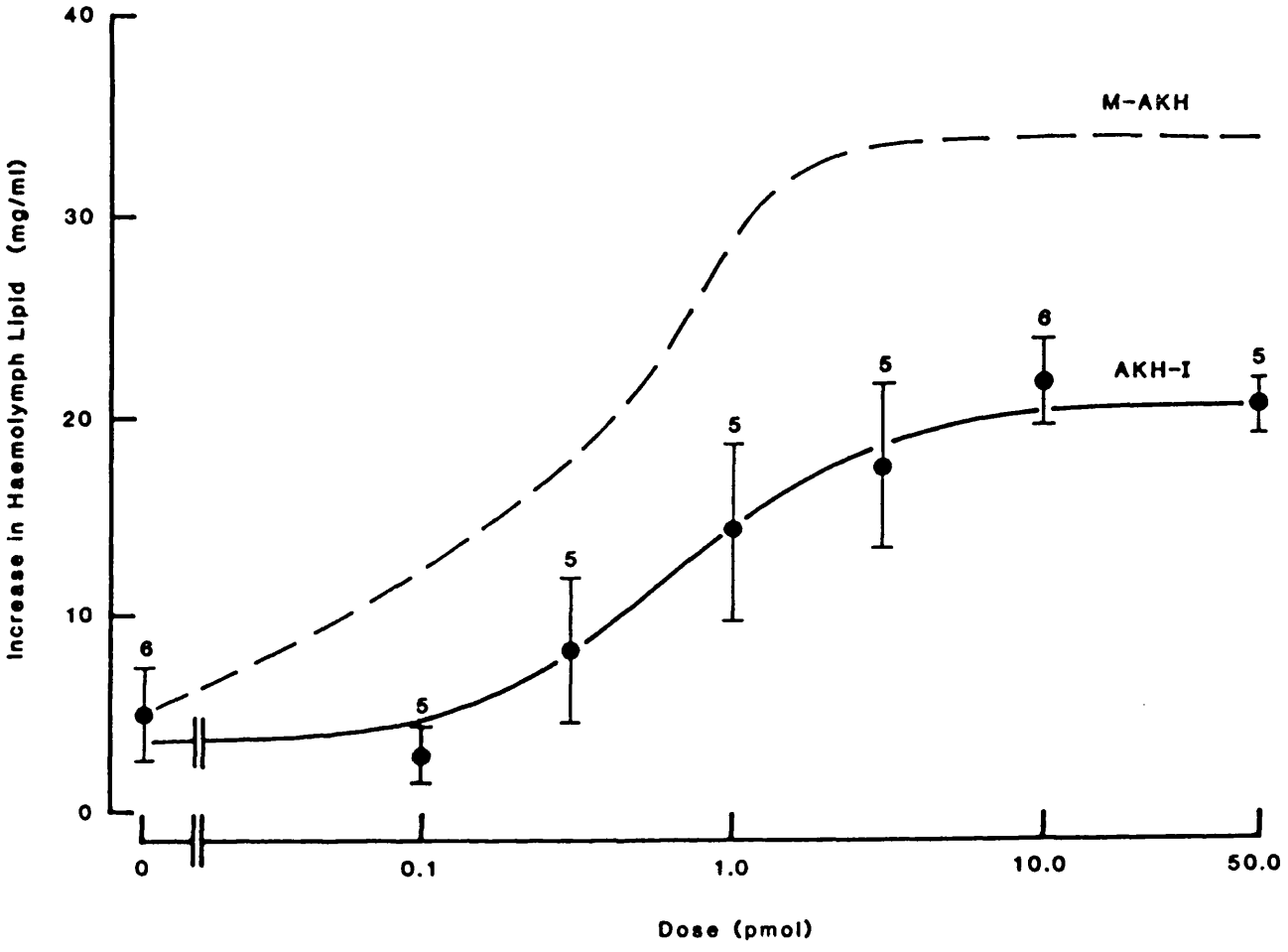


Fig. 5.2. Adipokinetic assay of synthetic AKH-I in adult Manduca. Details as for Fig. 5.1.

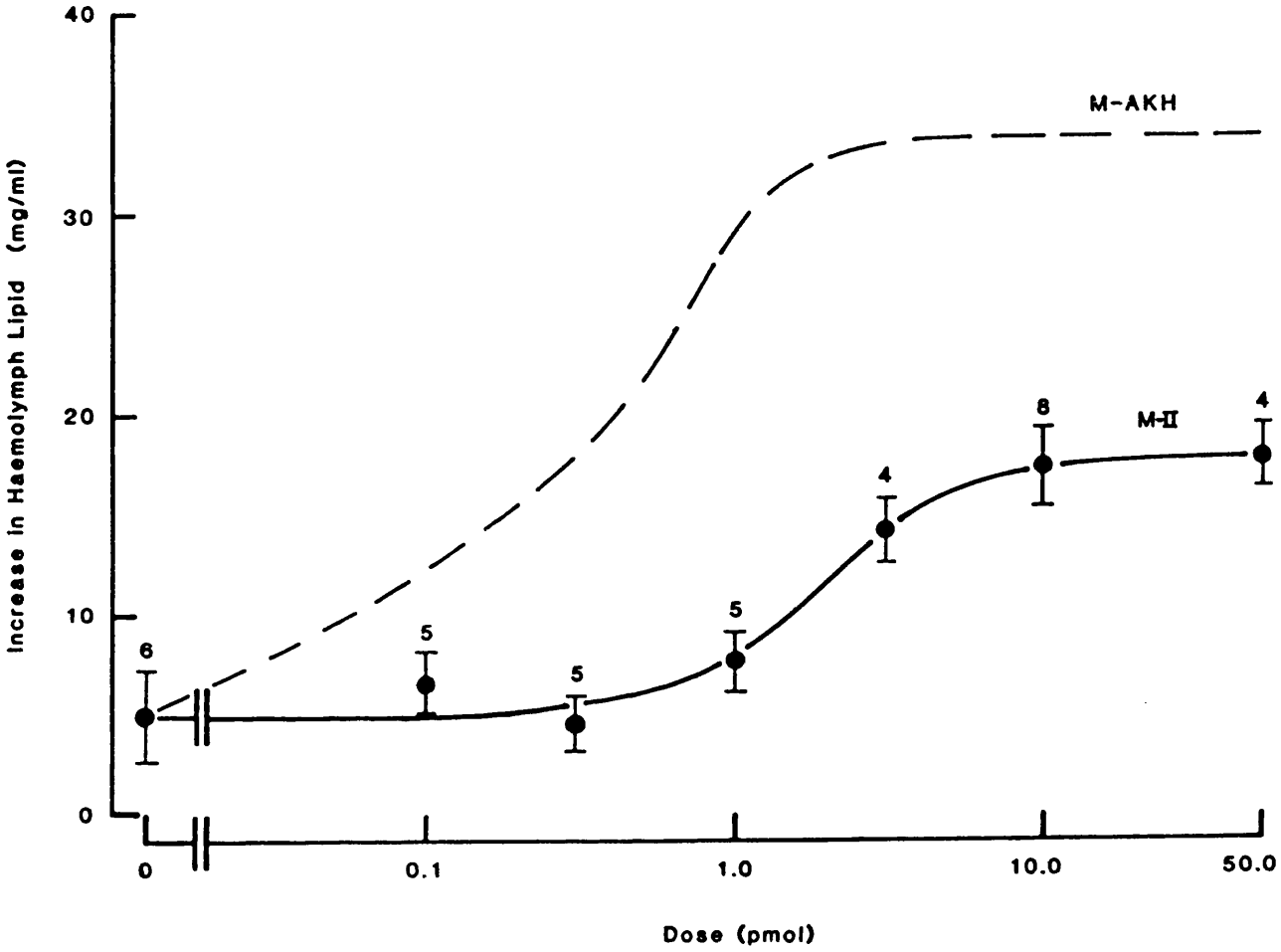


Fig. 5.3. Adipokinetic assay of synthetic M-II in adult Manduca. Details as for Fig. 5.1.

Table 5.2. The adipokinetic effect of 20 pmol of AKH/RPCH peptides alone and co-injected with 2 pmol of M-AKH.

Peptide	Adipokinetic Response (mg/ml)	
	20 pmol Test Peptide	20 pmol Test Peptide + 2 pmol M-AKH
Control (no test peptide)	4.7 ± 1.9 (5)	32.3 ± 1.5 (14)
HTF-II	20.8 ± 1.5 (6)	22.0 ± 2.0* (7)
M-II	18.8 ± 3.1 (9)	33.8 ± 1.7 (9)
AKH-I	22.6 ± 1.9 (5)	24.7 ± 3.0* (6)
RoI	5.5 ± 3.9 (5)	30.5 ± 2.4 (7)
HTH	8.2 ± 2.2 (5)	34.2 ± 2.3 (5)
M-I	7.1 ± 1.8 (5)	30.8 ± 2.1 (5)
RoII	2.6 ± 2.7 (5)	31.1 ± 2.2 (5)
AKH-II(S)	4.7 ± 2.4 (5)	32.6 ± 3.6 (5)
AKH-II(L)	5.3 ± 3.5 (5)	34.8 ± 2.6 (5)
RPCH	5.7 ± 2.3 (6)	28.8 ± 1.5 (5)

Control injection was 10 µl of 10% acetonitrile. Values indicate the mean response ± S.E. for the number of replicates indicated in parentheses.
* indicates a significant difference between the co-injected response and the response to M-AKH alone (t-test, $p > 0.01$).

CHAPTER 6. ENZYMATIC DEGRADATION OF M-AKH BY HAEMOLYMPH

INTRODUCTION

Neuropeptide inactivation has been extensively investigated in vertebrates during the last ten years. Although inactivation by non-enzymatic processes, such as diffusion, re-uptake and peptide-receptor internalisation cannot be discounted, the majority of studies have concentrated on the hydrolysis of neuropeptides by membrane-bound and soluble peptidases (Bauer, 1985; McKelvy and Blumberg, 1986). These studies have shown that peptidase specificity is usually determined by the localisation of the enzyme rather than the specificity of the enzyme for its substrate (Kenny, 1986).

In comparison with vertebrate studies our understanding of insect neuropeptide inactivation is very limited. The first detailed report on the inactivation of an insect neuropeptide was provided by Starratt and Steele (1984) who studied the *in vivo* inactivation of the cockroach pentapeptide proctolin (Arg-Tyr-Leu-Pro-Thr) by haemolymph. Using [^{14}C-Tyr^{2}]proctolin they demonstrated the degradation of proctolin by an aminopeptidase. Quistad *et al.* (1984) used tritiated proctolin to investigate the degradation of the peptide by a number of different tissues. They observed rapid proctolin degradation by all the tissues studied. A subsequent *in vitro* study of proctolin degradation by cockroach haemolymph indicated different cleavage sites depending on the pH of the haemolymph preparation (Steele and Starratt, 1985). Regardless of the exact mechanism it seems clear that proctolin is degraded in

the haemolymph. Proctolin is known to be released close to its sites of action rather than being a circulating neurohormone (O'Shea and Adams, 1986) and a locust neural membrane preparation has been shown to contain a proctolin-degrading aminopeptidase (Isaac, 1987).

The degradation of AKH/RPCH peptides remains an area of only rudimentary understanding. Mordue and Stone (1978) suggested that the Malpighian tubules were an important site for AKH-inactivation in the locust. The first detailed report on AKH/RPCH degradation was provided by Siegert and Mordue (1987) who demonstrated the degradation of AKH-I by Malpighian tubules using RP-HPLC to analyse the fragments of the degraded peptide. Baumann and Penzlin (1987) reported the rapid inactivation of the cockroach peptide, neurohormone D (which is known to be identical to the AKH/RPCH peptide, M-I) by intact and homogenized Malpighian tubules. These workers suggested that the active uptake of the peptide by tubule cells was followed by degradation by intracellular proteases. Isaac (1988) has recently characterised a neutral metalloendopeptidase from locust neural membranes which degrades AKH-I *in vitro*. Despite these findings it is not known where or how AKH/RPCH peptides are inactivated *in vivo*.

I have investigated the degradation of M-AKH by *Manduca* haemolymph *in vitro* and have obtained a partial characterisation of the enzyme involved.

MATERIALS AND METHODS

Initial Preparation of Haemolymph

Haemolymph was obtained from water-anaesthetised day 4, fifth instar larvae by cutting the abdominal horn and collecting the haemolymph in polypropylene tubes containing a few crystals of phenylthiourea (PTU). The haemolymph from 3-4 larvae was pooled and dialysed overnight against 0.1 M Tris/HCl, pH 7.5 (0.01 M Tris/HCl, pH 7.2 was used in later experiments). The dialysed haemolymph was either diluted in 0.1 M Tris/HCl, pH 7.5 and incubated with peptide as described below or further purified by ion-exchange chromatography.

Partial Purification of the Haemolymph Enzyme

Dialysed haemolymph (4 ml) was loaded onto a DEAE-Sephacel ion exchange column (10 x 0.5 cm in a BioRad disposable column) which had been equilibrated in 0.01 M Tris/HCl, pH 7.2. The column was washed with 0.05 M NaCl in the starting buffer mentioned above at a flow rate of 10 ml/h (controlled by an LKB 2132 Microperpex peristaltic pump). The enzyme was eluted by a gradient of 0.05-0.275 M NaCl in 50 ml of the starting buffer. The eluate was monitored at 280 nm by an Isco Model UA-5 absorbance monitor. Fractions were collected over 15 min intervals (2.5 ml each) using an Isco Model 1200 Pup Golden Retriever fraction collector. A 50 µl aliquot from each fraction was assayed for enzyme activity.

Enzyme Incubation

50 µl of diluted, dialysed haemolymph or partially purified

enzyme was added to a 1.5 ml polypropylene tube containing 1-5 µg of lyophilised M-AKH. For inhibitor experiments the purified enzyme was diluted with inhibitor solution and preincubated at room temperature for 20-30 min before a 50 µl aliquot of the mixture was incubated with lyophilised peptide. For the pH optimization experiment partially purified enzyme from the two most active fractions was pooled and applied to a series of pasteur pipettes containing Sephadex G-25 equilibrated at the appropriate pH by 0.01 M phosphate buffer (pH 6.0-pH 7.0) or 0.2 M Tris/HCl (pH 7.5-pH 9.0). The eluate between 10 and 30 drops which contained the enzyme was collected and a 50 µl aliquot of this was incubated with 1 µg of lyophilised peptide.

Incubations were performed at 26°C and usually lasted for 2-3 h with the exception of the time-course experiments and inhibitor studies (in which the incubation period was extended to 4 h to compensate for the dilution of the enzyme). Termination of the incubations was achieved by the addition of 1 ml of 0.1% TFA and the samples were then stored at -20°C.

HPLC Analysis of Enzyme Activity

Each sample was thawed and loaded onto a primed C_{18} SepPak cartridge. The sample was washed with 10% acetonitrile (2 ml), 60% acetonitrile (2 ml) and 100% acetonitrile (2 ml). The material eluted between 10% and 60% acetonitrile was collected in a polypropylene tube and freeze-dried. The dried sample was resuspended in 10 µl of 10% acetonitrile and injected onto a Spherisorb C_{18} 5 µm HPLC column (25 x 0.46 cm).

The solvents used for HPLC were 0.1% TFA (Buffer A) and 0.1% TFA in acetonitrile (Buffer B). The gradient used for initial studies of AKH degradation was 10% B (0-5 min); 10-60% B (5-30 min); 60% B (30-35 min) and 60-10% B (35-40 min). For the determination of the cleavage site the gradient was 15-40% B (0-20 min); 40-60% B (20-30 min); 60% B (30-35 min) and 60-15% B (35-40 min). The flow rate was 1 ml/min and the eluate was monitored at 210 nm. The peaks from the cleavage site experiment were collected manually, lyophilised in acid-washed pyrex tubes and amino acid analysed as described in Chapter 2.

Separation of Plasma from Cells

The method used for the separation of haemolymph plasma from cells was developed by Mead et al. (1986). Fifth instar larvae weighing 2.5-4.0 g were washed with distilled water, blotted dry and then chilled on ice. Each larva was swabbed with 70% ethanol prior to injection with 1 ml of ice cold anticoagulant solution (0.098 M NaOH, 0.146 M NaCl, 0.017 M EDTA and 0.041 M citric acid, pH 4.5).The larvae were returned to room temperature for 2 min and handled to encourage the even distribution of anticoagulant within the animal. Haemolymph was collected, following removal of the abdominal horn, in a chilled 1.5 ml polypropylene tube containing 500 µl of cold anticoagulant. The samples were spun in an MSE MicroCentaur at 12,000 g for 3 min. The supernatant (diluted plasma) was removed and dialysed overnight against 0.1 M Tris/Hl, pH 7.5. The pellet was washed with anticoagulant solution, resuspended in 0.1 M Tris/HCl, pH7.5, sonicated for 5 min and then

dialysed overnight. A 50 µl aliquot from each of the pellet and supernatant samples was incubated with peptide in order to determine the enzyme activity present in the cells and the plasma.

Molecular Weight Estimation

100 µl of dialysed haemolymph was injected onto a Sephacryl S-300 gel filtration column equilibrated in 0.1 M sodium phosphate buffer, pH 7.5. The sample was eluted at a flow rate of 6 ml/h and fractions were collected over 4 min intervals (400 µl each). The eluate was monitored at 280 nm. 50 µl aliquots from selected fractions were assayed for M-AKH degrading activity. The column was calibrated with the following molecular weight standards: Vitamin B_{12} (M.Wt. 1,350); cytochrome C (13,000); ribonuclease (13,700); haemoglobin (64,500); lactate dehydrogenase (146,000) and immunoglobulin G (158,000). The void volume was determined with blue dextran.

Effect of Substrate Concentration on Enzyme Activity

The most active fractions from an ion-exchange enzyme purification were pooled and used in the preparation of samples containing various concentrations of M-AKH. The samples were incubated at 26°C for 0, 30, 60 and 120 min, respectively. Termination of the incubations was achieved by the addition of 1 ml of 0.1% TFA. The samples were then processed through a SepPak cartridge and freeze-dried. The dried samples (duplicates for each time point)were resuspended in RIA incubation buffer and diluted appropriately in the buffer so that a 50 µl aliquot of each sample

contained a quantifiable amount of peptide (in theory). The samples were then assayed as described in Chapter 3. The amount of labelled peptide displaced in each tube indicated the amount of M-AKH in the sample and this figure was used to calculate the initial rate of degradation.

Other Sample Preparations

In order to investigate whether the haemolymph M-AKH degrading activity was enzymic in nature a sample of dialysed blood was divided into two portions. One portion was placed in a boiling water bath for 3 min and then cooled and centrifuged. The other portion was not boiled but was otherwise treated in the same way. 50 µl aliquots from the boiled supernatant and the unboiled sample were removed and assayed.

A preliminary study of the M-AKH degrading activity of whole Malpighian tubules was performed. Tubules were excised from a fifth instar larva and washed repeatedly in Manduca saline. The tubules were then incubated in 1.2 ml of saline at pH 7.0 with 12 µg of M-AKH. At appropriate times during the incubation a 200 µl aliquot of saline was removed and mixed with 200 µl of methanol to terminate any degradation. The terminated sample was centrifuged at 14,000 r.p.m. in an Eppendorf 5415 microcentrifuge for 5 min. The supernatant was then removed, diluted with 2 ml of 0.1% TFA and processed through a SepPak cartridge as described previously. The activity of the tubules was compared with that of a sample of dialysed haemolymph processed in a similar manner.

RESULTS

M-AKH was shown to be degraded by haemolymph from Manduca larvae when analysed by HPLC following the incubation of dialysed haemolymph with the peptide at a high concentration (5×10^{-5} M, Fig. 6.1). A comparison of the M-AKH degrading activity of dialysed haemolymph with that of intact Malpighian tubules, incubated in Manduca saline at pH 7.0, indicated little difference between the two tissues in their ability to degrade M-AKH over 30 min. However as the incubation period was extended the haemolymph samples appeared to be more effective than the intact tubules (Fig. 6.2). The time course for the degradation of M-AKH by diluted, dialysed haemolymph indicated a half-life for M-AKH of approximately 150 min (Fig. 6.3). On this basis the haemolymph M-AKH-degrading activity was assessed in subsequent experiments following a 3 or 4 h incubation in dialysed haemolymph. The M-AKH degrading activity was completely abolished by boiling the haemolymph before the assay (Fig. 6.4a). The rate of degradation was also dependent on the ambient temperature (Fig. 6.4b), with a Q_{10} of about 2.5. The increase in degrading activity with temperature began to fall away at temperatures above 30°C. These results suggested that the M-AKH degrading activity was probably enzymic in nature, so an attempt was made to characterise the M-AKH degrading enzyme(s) from Manduca haemolymph.

The enzyme activity was located in the 'plasma' fraction of the haemolymph rather than in the cells (Fig. 6.5). Samples of haemolymph were collected from insects at various stages of development, dialysed and then assayed to determine the levels of

enzyme activity. There appeared to be little variation in the level of enzyme activity during development of the insect (Fig. 6.6). Although activity in pharate adult haemolymph appeared to be slightly greater than in larval haemolymph, the small sample size (n = 2) precludes any confidence in this conclusion. Since adult haemolymph did not appear to be very much more active than larval haemolymph, and since fifth instar larvae possess considerably more haemolymph than adult moths, larval haemolymph was used in all subsequent experiments.

The molecular weight of the enzyme was estimated by gel filtration of dialysed, larval haemolymph (Fig. 6.7). The elution volume (V_e) for the M-AKH degrading activity was divided by the void volume of the column (V_o) to obtain a ratio which could be compared with the ratios for standards of known molecular weight to obtain an estimate of the molecular weight of the enzyme. The active component appeared to have a molecular weight of approximately 66 kDa.

Partial purification of the enzyme was achieved by DEAE-Sephacel anion exchange chromatography of dialysed larval haemolymph (Fig. 6.8a). The time-course for M-AKH degradation by the most active fraction (Fig. 6.8b) indicated a half-life of approximately 75 min, closer to the expected half-life of M-AKH *in vivo* (less than 60 min, see Chapter 3). Additional characteristics of the partially purified enzyme were then investigated. The enzyme had a neutral pH optimum (pH 7.0-7.5, Fig. 6.9) notably higher than the pH of larval haemolymph (pH 6.7). A number of

inhibitors were assayed for their effect on the M-AKH degrading enzyme (Table 6.1). The most effective inhibitors at a concentration of 1 mM were the metal-chelating agents EGTA and 1,10-phenanthroline (87% and 99% inhibition, respectively). The sulphydryl inhibitor p-chloromercuribenzoate (PCMB) also appeared to be an effective inhibitor at the test concentration. In contrast, another sulphydryl inhibitor, N-ethylmaleimide, displayed only a weak inhibitory effect. The precise mode of inhibition by PCMB therefore remains uncertain. The serine protease inhibitor phenylmethanesulphonyl fluoride (PMSF) displayed a limited inhibitory effect (22%) on the haemolymph enzyme, however soybean trypsin inhibitor (SBTI) had no significant effect and in a preliminary experiment the chymotrypsin inhibitor TPCK also failed to inhibit the enzyme in dialysed haemolymph (data not shown). None of the other inhibitors tested caused a significant reduction in the activity of the haemolymph enzyme. A number of other AKH/RPCH peptides were incubated with samples of partially purified enzyme in order to investigate the specificity of the enzyme (Table 6.2). All the peptides tested were degraded to a similar extent during a 2 h incubation with the enzyme.

In an attempt to determine the primary cleavage site in the degradation of M-AKH the fragment peaks isolated by RP-HPLC were collected and analysed for their amino acid composition (Fig. 6.10). The results suggested that the first product peak was the N-terminal fragment pGlu-Leu-Thr-Phe-Thr. The second product peak appeared to be composed largely of glycine but its precise identity remains uncertain as smaller quantities of threonine, serine,

glutamine and leucine were also present.

The effect of substrate concentration on the rate of M-AKH degradation in vitro was determined in a preliminary experiment using the M-AKH RIA. The amount of peptide present in each assay tube was used to calculate the initial rate of degradation in terms of the amount of M-AKH lost during the first hour of incubation. Fig. 6.11 shows that the partially purified enzyme was not saturated with M-AKH, even at a concentration of 10^{-5} M. The rate of degradation was extremely slow at peptide concentrations below 10^{-7} M. Even if the rate of degradation of 10^{-5} M peptide represents the maximum initial rate, the peptide concentration at which the rate of degradation is 50% of the maximum rate (known as the Michaelis constant or K_m) would be at least 10^{-6} M, some four orders of magnitude higher than the concentration of M-AKH in the haemolymph of starved larvae reported in Chapter 3.

DISCUSSION

Studies on the inactivation of AKH/RPCH family peptides have lagged behind investigations of other aspects of the physiology and biochemistry of these peptides. Cheeseman et al. (1976) speculated that the half-life of AKH-I in locusts would be around 20 min during rest or flight. Preliminary experiments on the degradation of AKH-I by locust Malpighian tubules were performed using semi-isolated tubule preparations (Mordue and Stone, 1978). AKH-I was rapidly removed from the bathing fluid and the secreted fluid lacked adipokinetic activity. The removal of the peptide from the bathing fluid appeared to occur at a constant rate of approximately

2-3 pmol/h regardless of the dose injected. More recently Siegert and Mordue (1987) investigated the degradation of AKH-I by homogenates of Malpighian tubules from Schistocerca. The hormone appeared to be completely destroyed (as defined by the disappearance of the AKH-I peak from HPLC traces) within 1 h when incubated with homogenate at 30°C. The breakdown products were shown to lack adipokinetic activity at a dose of 20 pmol/locust and their structures were determined by gas-phase sequencing. The primary cleavage appeared to be dependent on the activity of an endopeptidase at the bond between the [Pro^6] and [Asn^7] or the [Asn^7] and [Trp^8] residues. An aminopeptidase (possibly leucine aminopeptidase) and a carboxypeptidase (not carboxypeptidase A) were also present in the tubules. The means by which AKH-I entered the tubule cells was not determined. A number of other locust tissues (but not haemolymph) were also found to be capable of AKH-I degradation.

Loughton (1987) confirmed the inability of locust haemolymph to inactivate AKH-I, however he reported evidence for the involvement of the fat body in AKH-I inactivation. The fat body plasma membrane could not degrade AKH-I by proteolysis but Loughton suggested that protein synthesis was a required step in the inactivation of fat body membrane-bound AKH-I. Locust synaptic membranes have recently been shown to possess an endopeptidase capable of degrading AKH-I by cleavage of the bond between [Asn^4] and [Phe^5] (Isaac, 1988). This enzyme appears to be similar to the mammalian kidney endopeptidase 24.11 (Turner et al., 1985), however it is not known whether the locust enzyme has a physiological role in AKH-I

degradation.

The cockroach peptide M-I (also known as neurohormone D) was inactivated (as defined by the loss of cardioactivity) *in vitro* following incubation with intact cockroach Malpighian tubules or homogenates of them (Baumann and Penzlin, 1987). The active uptake of the peptide into tubule cells was demonstrated and the enzyme responsible for peptide inactivation was characterised as a metalloendopeptidase with serine or cysteine at the active site. These workers found no evidence for inactivation by any other tissues from *Periplaneta*.

Skinner *et al.* (1987) investigated the degradation of tritiated M-II (also known as CC-2) by haemolymph and homogenates of fat body and Malpighian tubule from *Periplaneta*. They found the half-life of the peptide to be around 1 h *in vitro* and *in vivo*, with very little degradation by haemolymph. It was suggested that the synthesis and secretion of M-II were more important than degradation in determining the circulating levels of the peptide.

My preliminary HPLC analyses indicated that haemolymph from larval *Manduca* was capable of degrading the native peptide, M-AKH *in vitro*. In contrast with previous work in other insects, peptide degradation by haemolymph appeared to be as effective as that observed following the incubation of peptide with intact Malpighian tubules. Experiments with boiled haemolymph and incubations at different ambient temperatures suggested that the degrading activity was probably due to a proteolytic enzyme. The characteristics of the enzyme were investigated using dialysed and partially purified haemolymph preparations. An assay of the

separated 'plasma' and 'cellular' fractions of the haemolymph indicated that the enzyme was a soluble protease rather than a membrane-bound or intracellular enzyme.

Ziegler (1984) reported developmental variation in the responsiveness of the Manduca fat body to in vivo injections of CC extract. He concluded that the variation was due to changes in the fat body rather than changes in the level of hormone synthesis or secretion. My results suggest that the variation in response is unlikely to be due to changes in the level of M-AKH degradation by haemolymph as the level of enzyme activity did not vary significantly during the late fourth and fifth larval instars and into adulthood.

The time course for the breakdown of M-AKH by the partially purified enzyme indicated a half-life of around 75 min for the hormone under the assay conditions. The half-life of M-AKH in starved Manduca larvae appeared to be around 60 min (as described in Chapter 3) so that the time-course data are consistent with a possible role for the haemolymph enzyme in M-AKH degradation. However the pH optimum experiment indicated that the enzyme would not be maximally active at the pH of larval haemolymph.

The haemolymph enzyme appeared to be similar, in terms of its molecular weight (66 kDa), pH optimum (7.0-7.5) and inhibition by metal-chelating agents, to a soluble metalloendopeptidase isolated from rat brain by Orlowski et al. (1983). These workers defined the amino acid requirements for a suitable peptide substrate. The peptide should have aromatic residues at positions P'_3 and P_1 or P'_3, P_1 and P_2 (where P_1 is the residue adjacent to the cleaved

bond on the N-terminal side, P_2 is next to P_1 on the N-terminal side and P'_3 is three residues away from the cleaved bond on the C-terminal side). Amino acid analysis of the fragments produced by enzymatic cleavage of M-AKH suggests that one of the primary products may be the pentapeptide pGlu-Leu-Thr-Phe-Thr. Cleavage at the bond between the [Thr^5] and [Ser^6] residues implies partial fulfilment of the substrate requirements defined by Orlowski et al. The P_2 and P'_3 positions are occupied by the aromatic residues [Phe^4] and [Trp^8], respectively, but the P_1 position is occupied by [Thr^5] which is not an aromatic amino acid. All the AKH/RPCH peptides sequenced to date posess a [Phe^4] and a [Trp^8] residue and all the peptides assayed with partially purified enzyme were degraded as effectively as M-AKH. However the fragments produced by the cleavage of non-M-AKH peptides have not been analysed so it would be premature to conclude that the peptides which were assayed were all cleaved in the same manner by a single enzyme.

Although the haemolymph may not be the only tissue containing M-AKH degrading activity, it is clear that Manduca differs from locusts and cockroaches in which the haemolymph has virtually no activity in this regard. The difference between these insects may be related to the rate of circulation of the haemolymph which is very slow in the caterpillar compared with that of the locust or the cockroach.

In order to evaluate the probable importance of the haemolymph enzyme an attempt was made to estimate the concentration of M-AKH at which enzyme activity was limited by lack of substrate. The data suggest that the K_m of the enzyme may be at least four orders of

magnitude higher than the concentration of M-AKH in the haemolymph of starved larvae. At best this would appear to suggest that the M-AKH degrading enzyme in Manduca larval haemolymph represents a low-affinity inactivation system rather than the principal means of peptide inactivation. However the haemolymph enzyme may not normally operate under equilibrium conditions and it is likely that additional haemolymph enzymes remove the products of the initial M-AKH cleavage increasing the effectiveness of the M-AKH cleaving enzyme. The pattern of RP-HPLC peaks from peptide samples incubated with partially purified enzyme was much simpler than that obtained from samples incubated with dialysed haemolymph, suggesting the presence of additional enzymes that further cleave the hormone fragments.

In conclusion, a neutral metalloendopeptidase has been identified in the haemolymph of larval Manduca. The enzyme appears to be similar to a soluble peptidase previously characterised from rat brain. The physiological role of this enzyme remains unclear.

Fig. 6.1. HPLC analysis of M-AKH degradation by dialysed larval haemolymph. Haemolymph from day 4, fifth instar larvae was dialysed against 0.2 M Tris/HCl, pH 7.5 and then diluted to 20% haemolymph. 50 µl aliquots of diluted haemolymph were incubated with lyophilised M-AKH (5 µg) for 0 h or 4 h. Incubations were terminated with 1 ml of 0.1% TFA and the mixture was SepPaked prior to HPLC on a Spherisorb 5 µm C_{18} column (25 cm x 0.46 cm). Solvent A was 0.1% TFA; solvent B was 0.1% TFA in acetonitrile. Gradient elution is indicated by the dotted line. Flow rate was 1 ml/min.

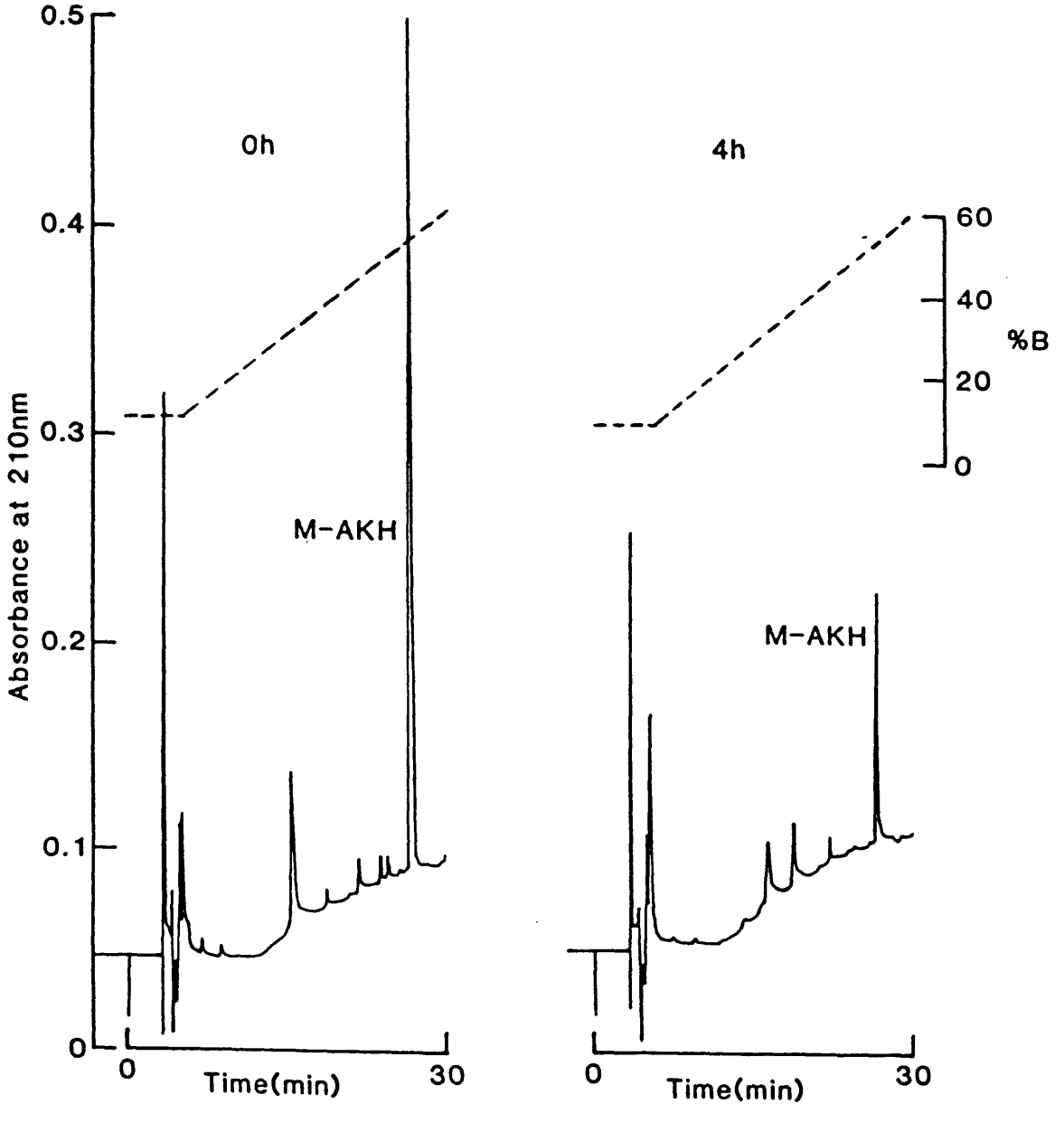
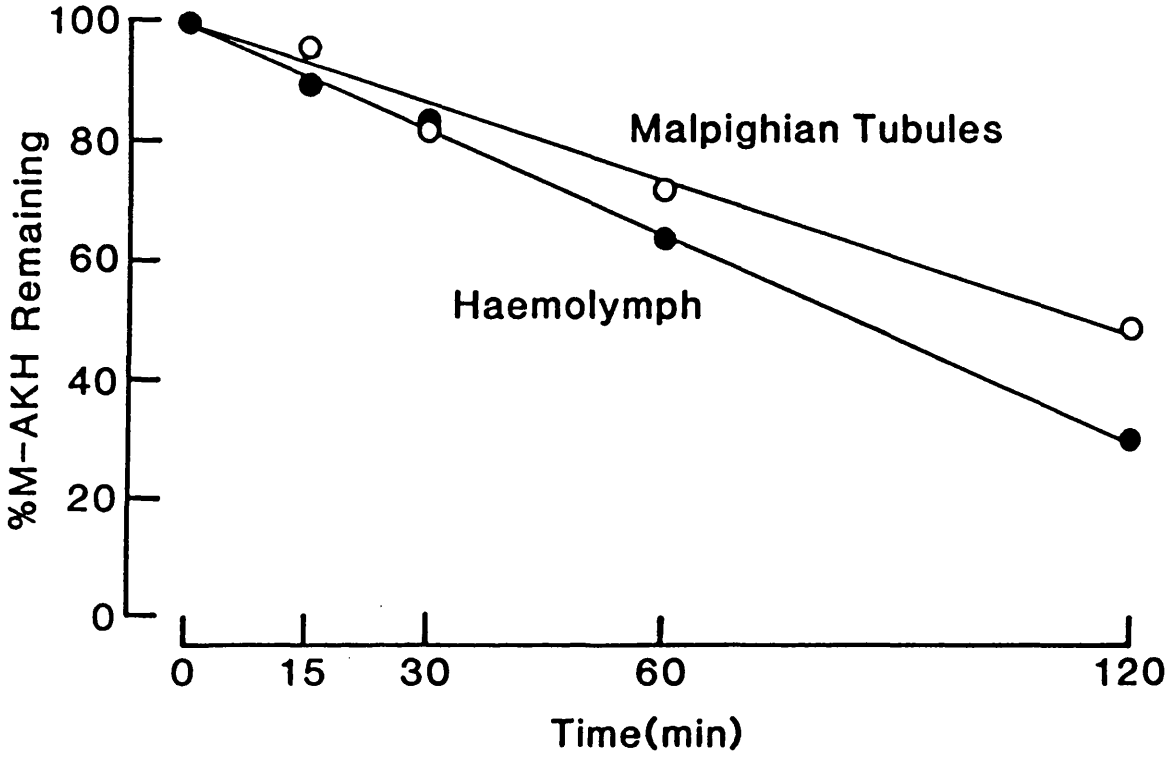

0.5
0.4
0.3
0.2
0.1
0
Absorbance at 210nm
0h
M-AKH
0
Time(min)
30
4h
M-AKH
0
Time(min)
30
60
40
20
0
%B

Fig. 6.2. In vitro degradation of M-AKH by intact Malpighian tubules (○) or dialysed haemolymph (●). Tubules from one insect (day 4, fifth instar larva) were incubated with 12 µg of M-AKH in 1.2 ml of Manduca saline, pH 7.0. 200 µl aliquots were removed at the times indicated and terminated with 200 µl of methanol. The terminated sample was centrifuged at 14,000 r.p.m. for 5 min, the supernatant removed, diluted with 0.1% TFA, and SepPaked prior to HPLC. 1.2 ml of dialysed haemolymph was also incubated with 12 µg of M-AKH and aliquots taken as above. HPLC analysis as described in Fig. 6.1. Each point represents the mean of duplicate samples, with the % M-AKH remaining determined by the M-AKH peak height.


100
80
60
40
20
0
%M-AKH Remaining
Malpighian Tubules
Haemolymph
0
15
30
60
120
Time(min)

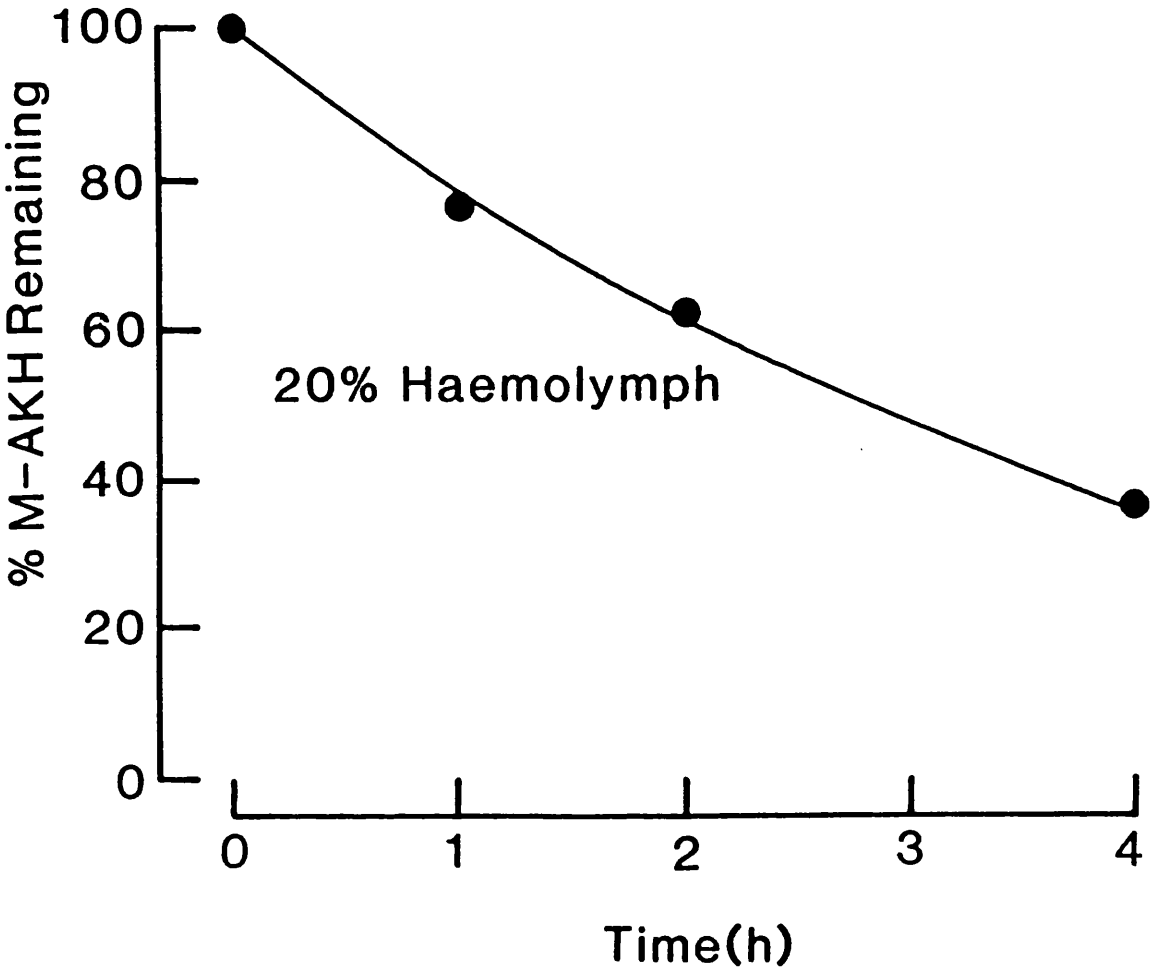
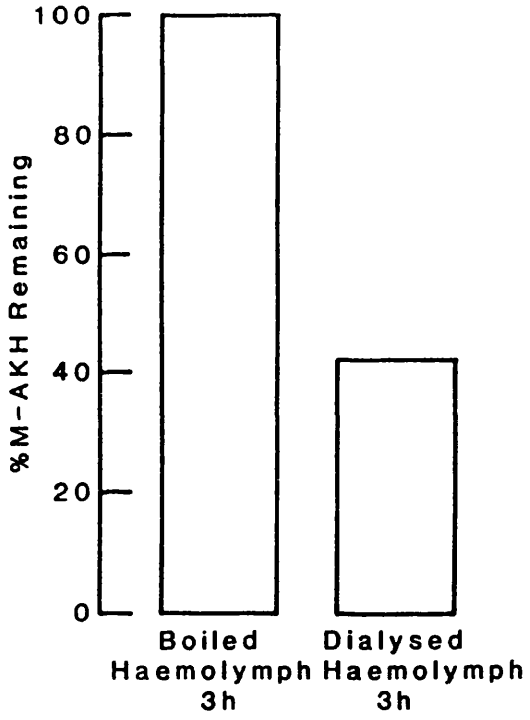
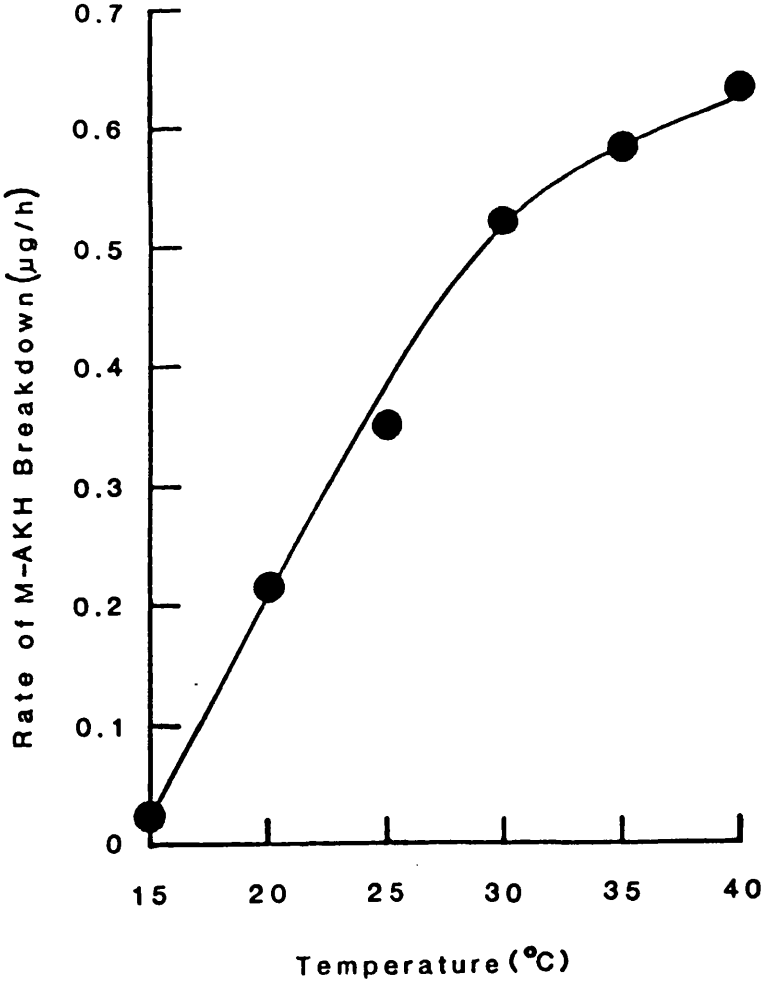


Fig. 6.3. Time course for the degradation of M-AKH by diluted, dialysed haemolymph. Degradation was assessed in terms of the change in the M-AKH peak height. Each point represents the mean of duplicate samples. Details of incubation and analysis as in Fig. 6.1.

Fig. 6.4. a) Comparison of M-AKH degradation by boiled and unboiled haemolymph from day 4 fifth instar larvae. Details as for Fig. 6.1. Results are the means of duplicate samples.

b) Effect of temperature on M-AKH degradation by dialysed haemolymph. Incubations were for 4 h at the temperatures indicated with other details the same as in Fig. 6.1. All points represent the means of triplicate samples. Rate of degradation was calculated from the peak height for M-AKH having calibrated the HPLC system with standard amounts of M-AKH.


100
80
60
40
20
0
%M-AKH Remaining
Boiled Haemolymph 3h
Dialysed Haemolymph 3h


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Rate of M-AKH Breakdown (µg/h)
15
20
25
30
35
40
Temperature (°C)

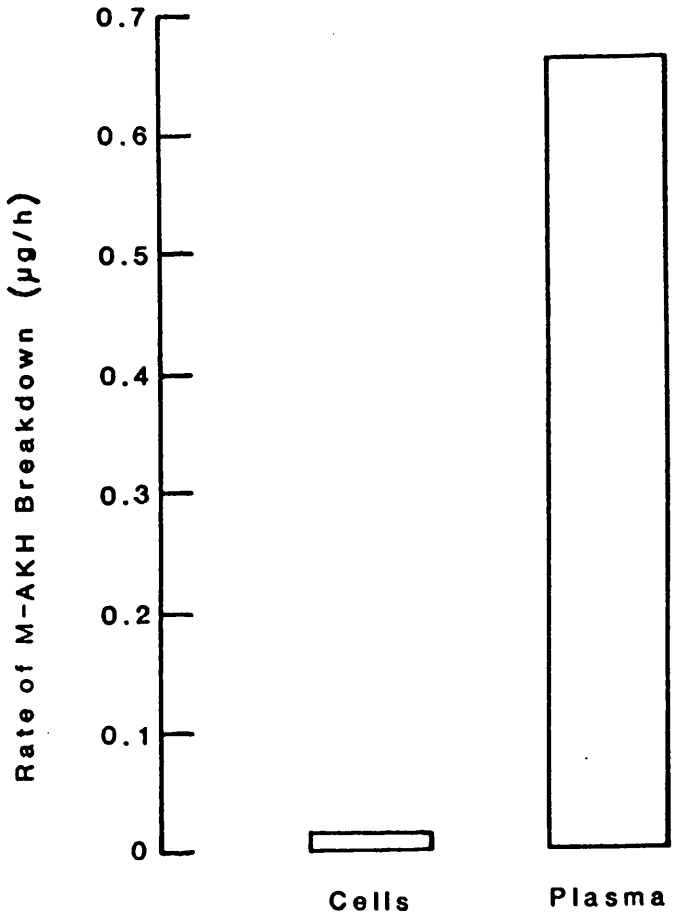


Fig. 6.5. Localisation of the M-AKH degrading enzyme in larval haemolymph. Plasma and cells were separated by the method of Mead et al. (1986). Results represent the means of duplicate samples. Rate determined as in Fig. 6.4.

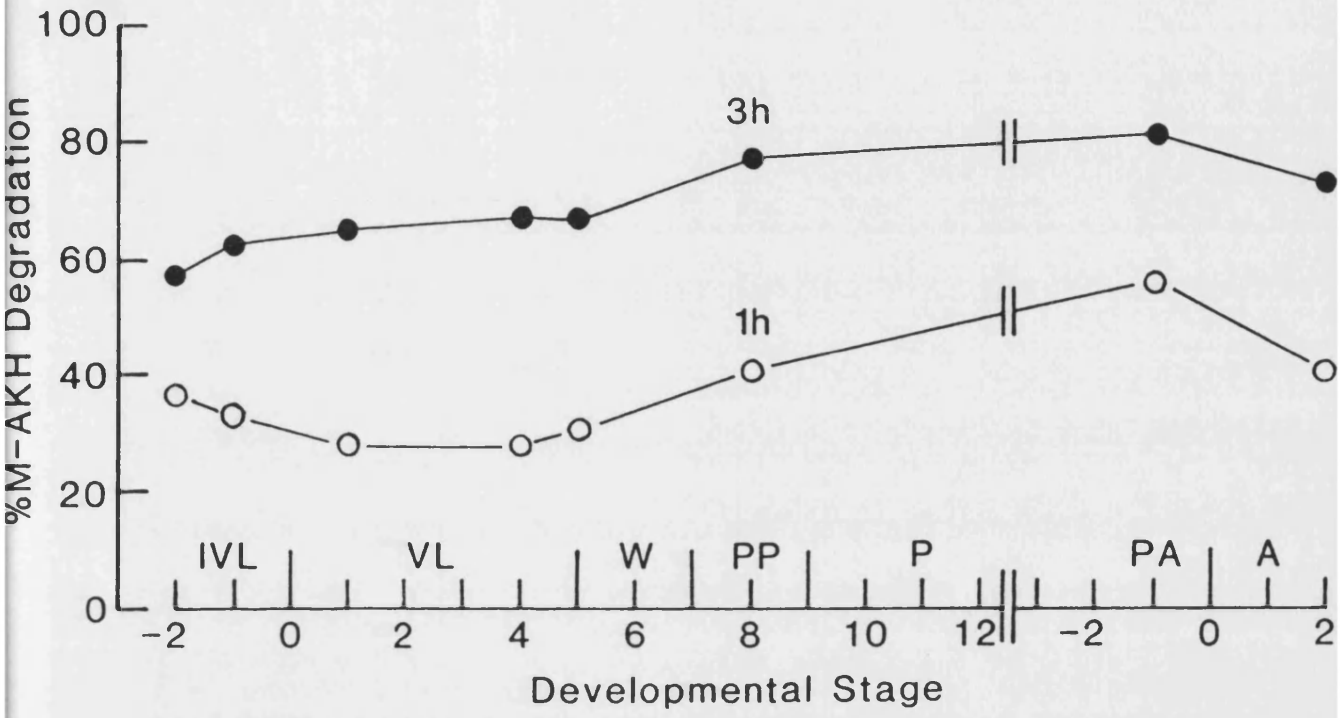


Fig. 6.6. Developmental variation in the levels of M-AKH degrading enzyme in Manduca haemolymph. Developmental stages indicated are fourth larval instar (IVL), fifth larval instar (VL), wandering phase (W), pharate pupa (PP), pupa (P), pharate adult (PA) and adult (A). Each point represents the mean of duplicate samples. Enzyme activity is expressed in terms of the percentage degradation of M-AKH after 1 h (○) or 3 h (●) incubations.

Fig. 6.7. Molecular weight determination for the M-AKH degrading enzyme. 100 µl of dialysed haemolymph was loaded onto a Sephacryl S-300 gel filtration column and eluted with phosphate buffered saline, pH 7.5. The column was calibrated with vitamin B_{12} (1,350); cytochrome c (13,000); ribonuclease (13,700); haemoglobin (64,500); lactate dehydrogenase (146,000) and immunoglobulin G (158,000). The void volume was determined with blue dextran.

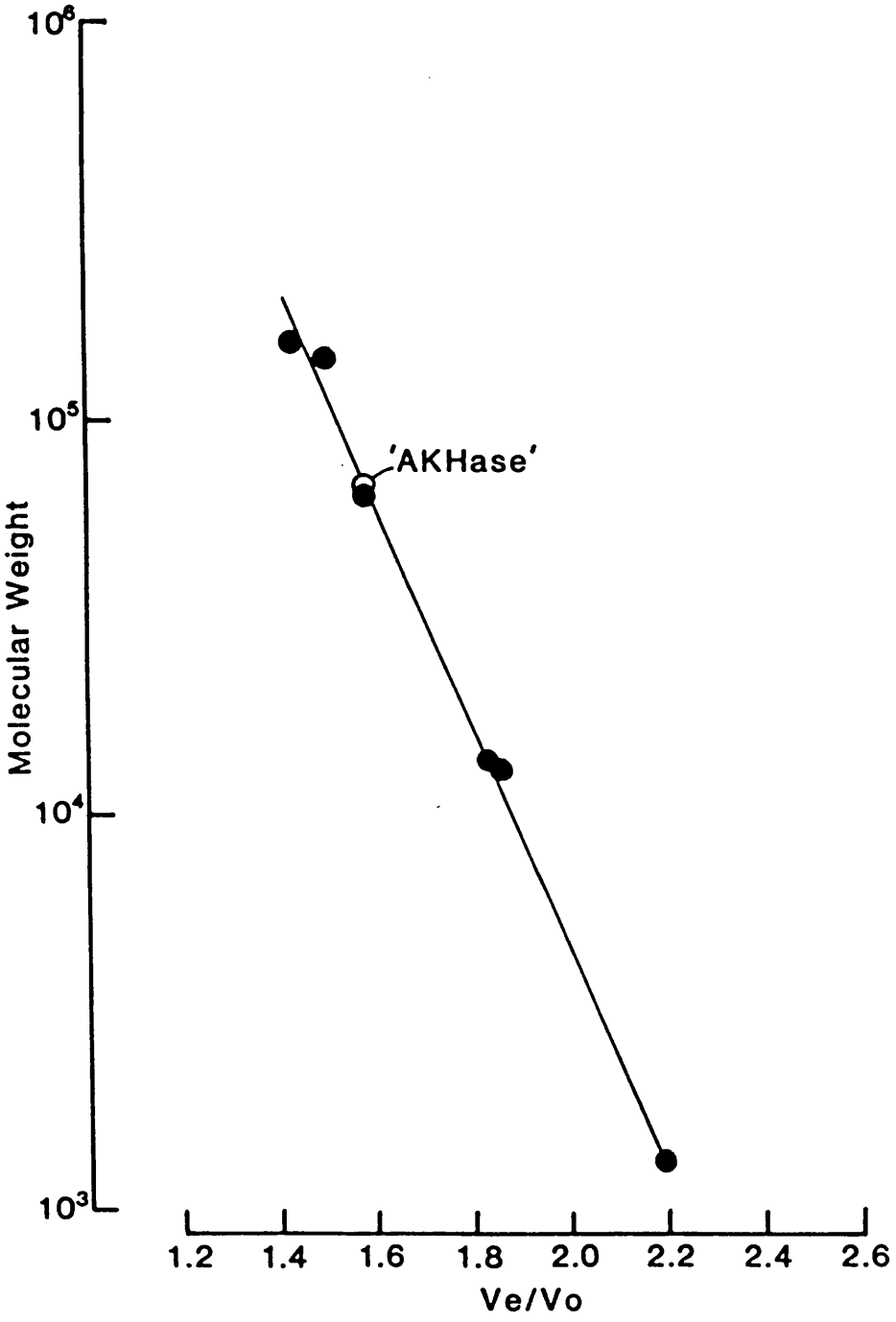
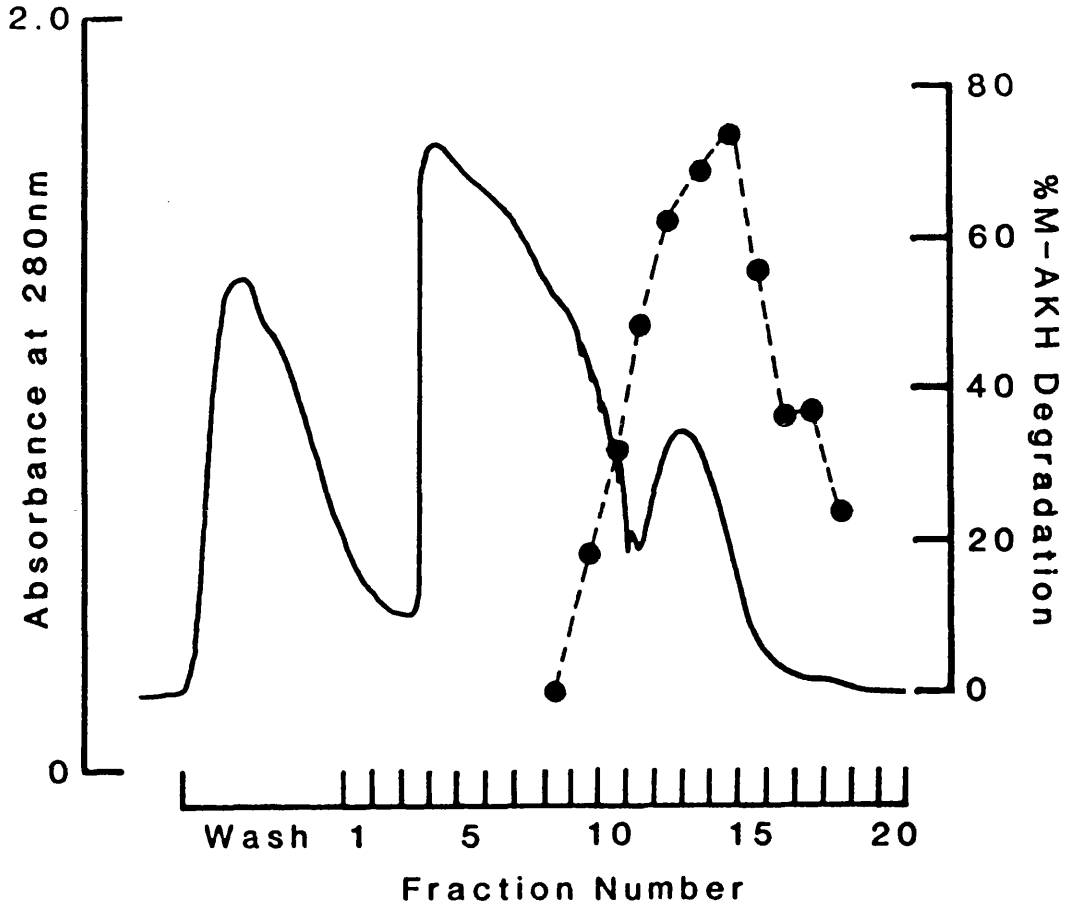
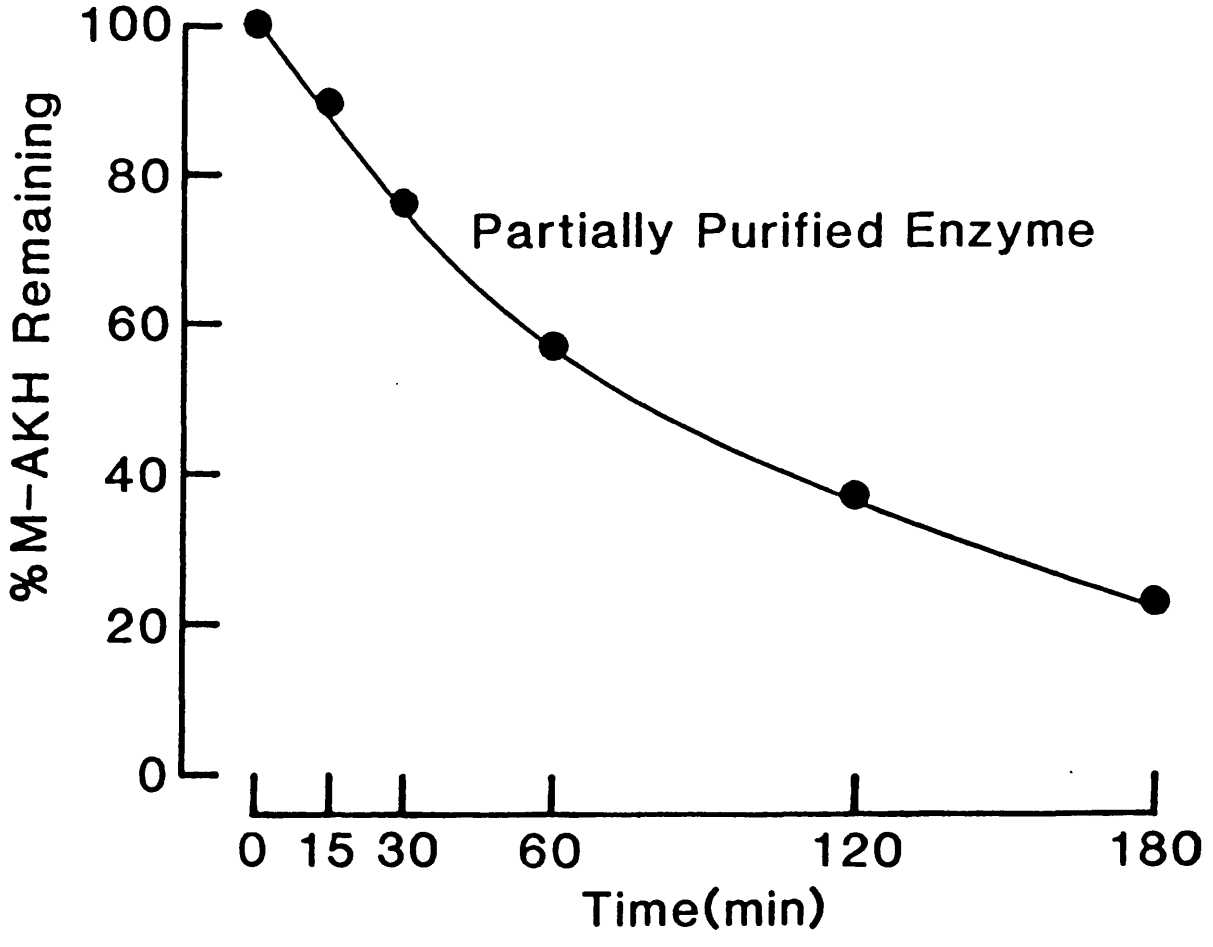

10^6
10^5
10^4
10^3
Molecular Weight
'AKHase'
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
Ve/Vo

Fig. 6.8. a) Partial purification of the haemolymph enzyme by DEAE-Sephacel ion exchange chromatography. 4 ml of dialysed haemolymph was loaded onto the column in 0.01 M Tris/HCl, pH 7.2 containing 0.05 M NaCl. The column was washed in this buffer and then eluted by a gradient of 0.05-0.275 M NaCl in the Tris buffer at a flow rate of 10 ml/h. Dotted line indicates enzyme activity of fractions around the final absorbance peak (no activity observed in earlier peaks). Each point represents the mean of duplicate samples. Each fraction was collected over a 15 min period (2.5 ml).

b) Time course for degradation of M-AKH by partially purified enzyme from fraction 14 in a). Details as for Fig. 6.3.


2.0
0
Absorbance at 280nm
80
60
40
20
0
%M-AKH Degradation
Wash
1
5
10
15
20
Fraction Number


100
80
60
40
20
0
%M-AKH Remaining
Partially Purified Enzyme
0
15
30
60
120
180
Time(min)

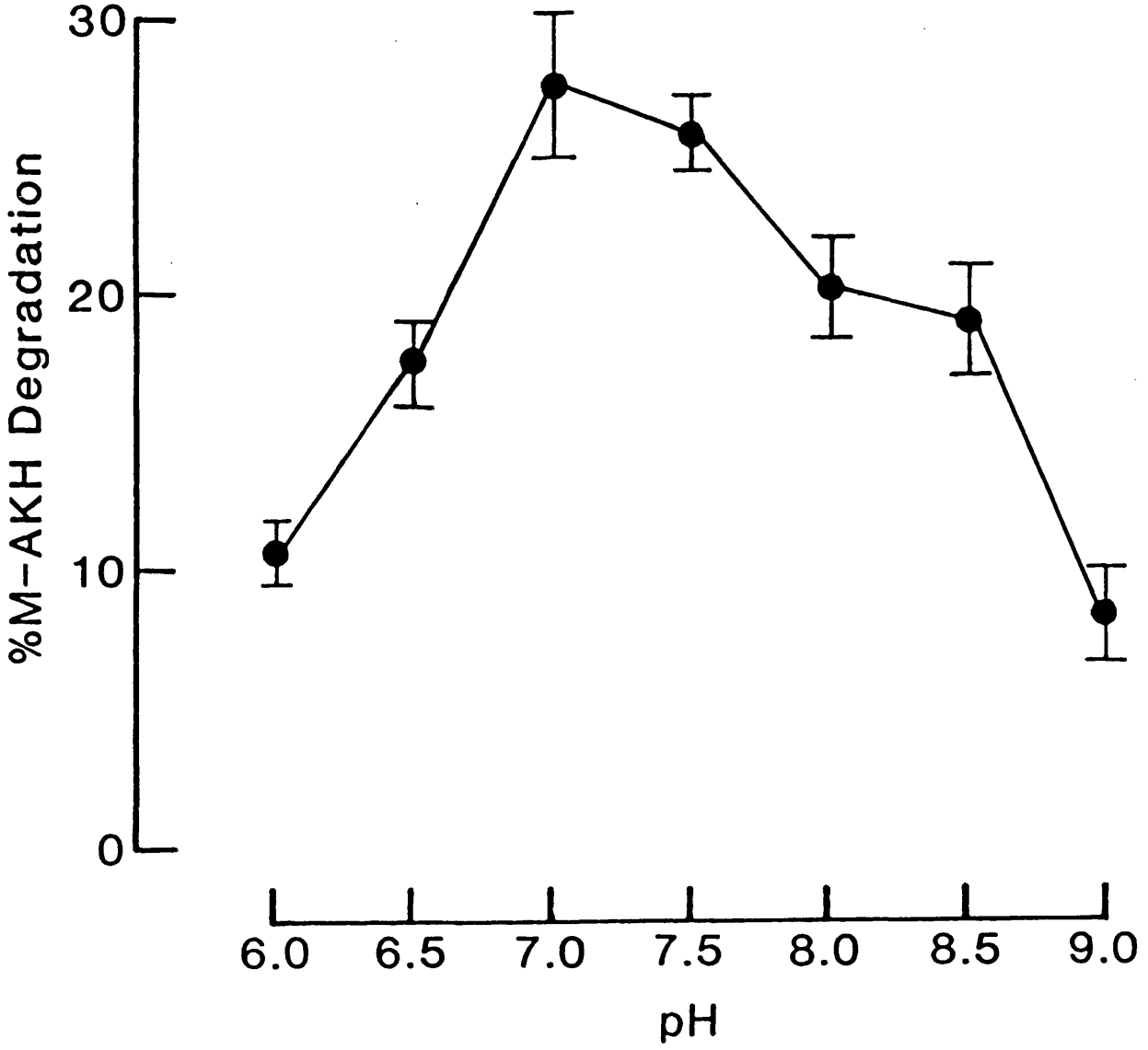


Fig. 6.9. Determination of the pH optimum for activity in the partially purified enzyme preparation. Samples of purified enzyme were buffer-exchanged using small columns containing Sephadex G-25 equilibrated at the appropriate pH. Each point and bar represents the mean ± S.E. for 4 samples.

Table 6.1. Effect of protease inhibitors on the activity of the partially purified enzyme.

Inhibitor	Concentration	% Inhibition
Phosphoramidon	0.46×10^{-3} M	0
S.B.T.I.	0.5 mg/ml	2
Leupeptin	0.5×10^{-3} M	3
Bestatin	1.45×10^{-3} M	4
N-Ethylmaleimide	1.0×10^{-3} M	13
PMSF	1.0×10^{-3} M	22
PCMB	1.0×10^{-3} M	70
EGTA	1.0×10^{-3} M	87
1,10-Phenanthroline	1.0×10^{-3} M	99

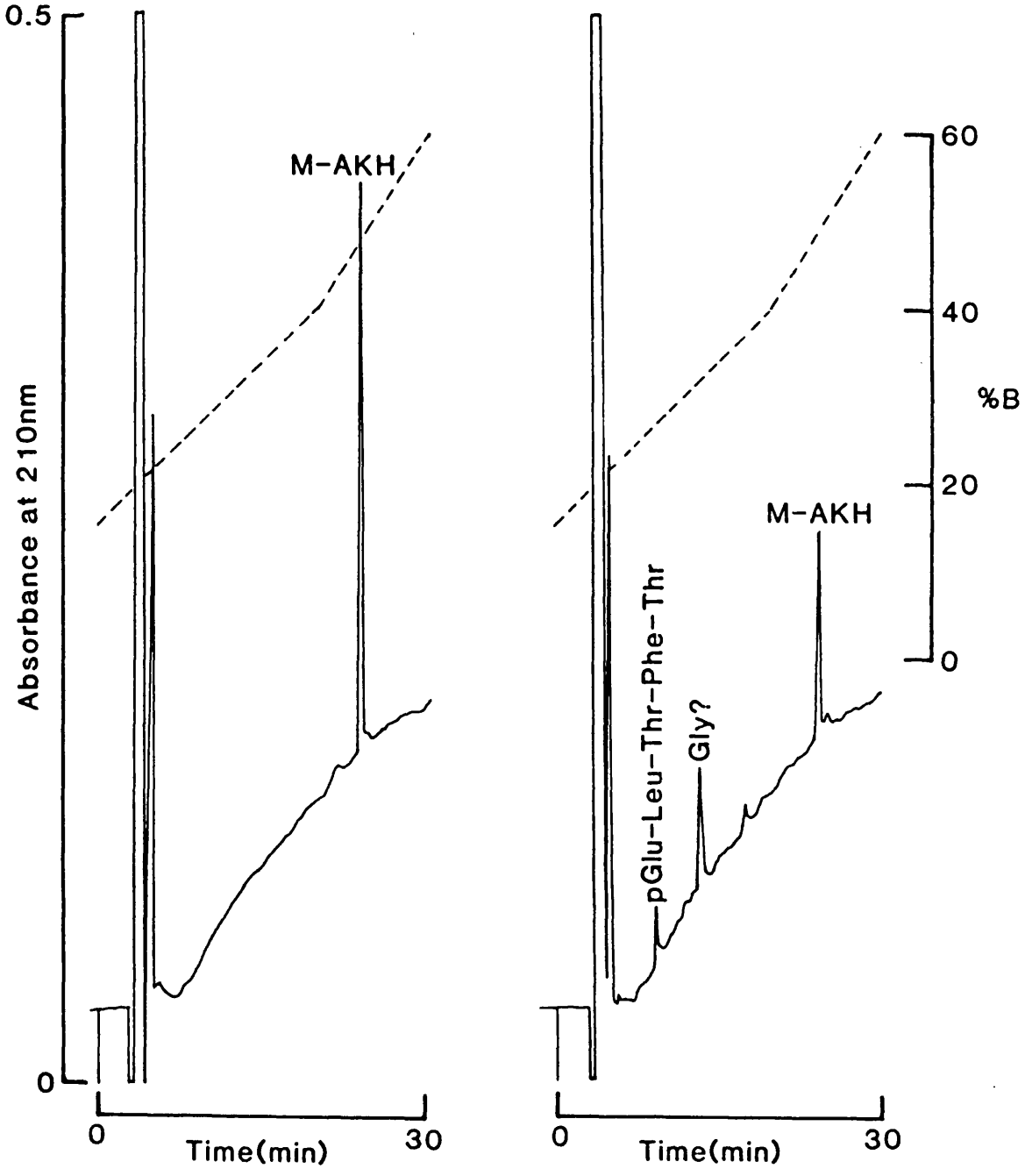
Enzyme and inhibitor were preincubated for 20 min and then added to lyophilised M-AKH and incubated for 4 h at 26°C. Results are expressed in terms of the % inhibition compared with control samples in which the enzyme was preincubated with buffer only.

Table 6.2. Investigation of the substrate specificity of the partially purified haemolymph enzyme.

Peptide	% Degradation over 3 h
M-AKH	99.2
AKH-I	99.5
AKH-II(S)	97.6
AKH-II(L)	95.9
RPCH	97.7
M-II	96.4
RoI	96.3

50 µl of enzyme was incubated with 1 µg of each peptide for 3 h at 26°C. Termination and HPLC analysis were performed as described in Fig. 6.1. Each value represents the mean of duplicate samples.

Fig. 6.10. Analysis of the primary cleavage site in the hydrolysis of M-AKH by partially purified enzyme. 40 µg of M-AKH was incubated for 2 h with 500 µl of enzyme. Aliquots from the reaction mixture were analysed by HPLC following termination and SepPak processing. Three peaks were collected, pooled and lyophilised. Chromatography details as in Fig. 6.1 with the gradient used indicated by the dotted line. Lyophilised material from the HPLC analysis was hydrolysed in 6 N HCl for 24 h *in vacuo* and analysed by a Hilger Chromaspek II analyser.


0.5
Absorbance at 210nm
0
M-AKH
0
Time(min)
30
60
40
%B
20
0
M-AKH
pGlu-Leu-Thr-Phe-Thr
Gly?
0
Time(min)
30

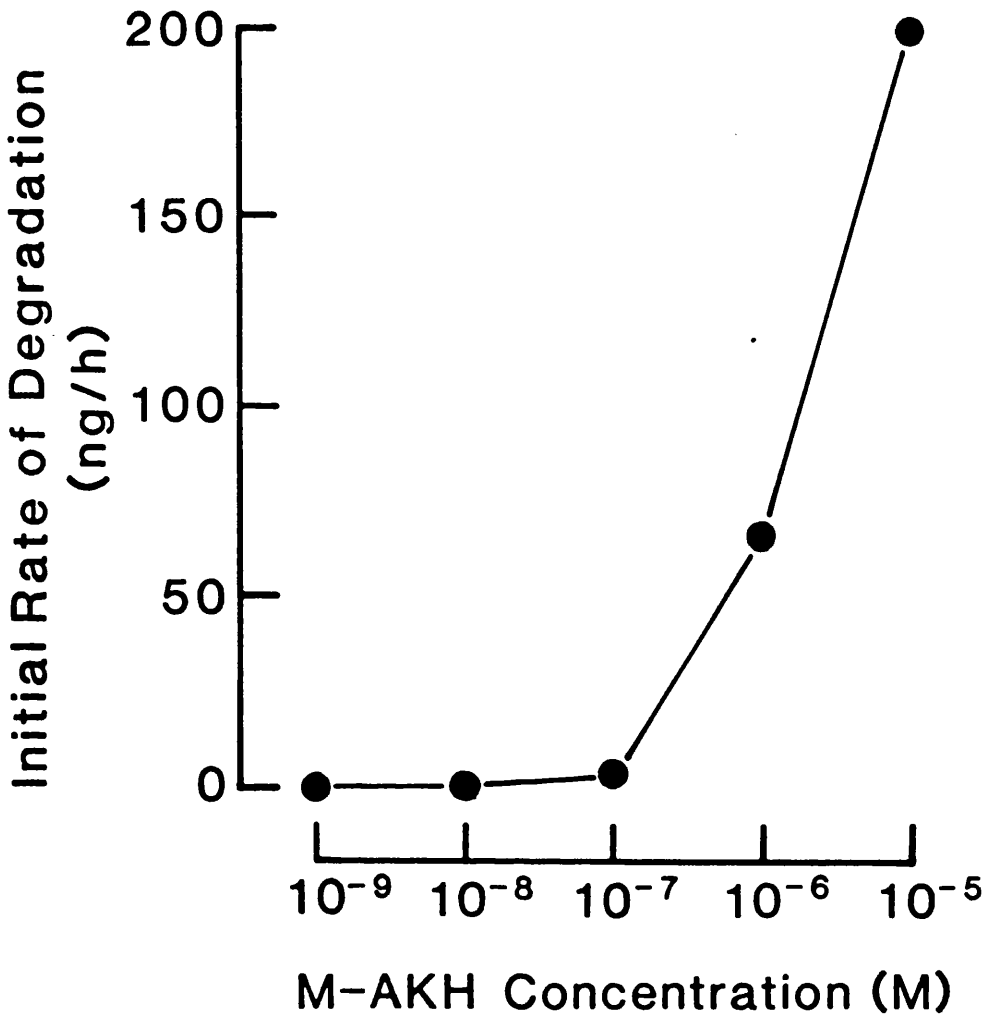


Fig. 6.11. Effect of substrate concentration on the activity of the M-AKH degrading enzyme. Partially purified enzyme was incubated with various concentrations of peptide. The amount of peptide degraded during the first hour was calculated from the RIA determination of the amount of peptide remaining. The rate of degradation during the first hour is shown. Each point is the mean of duplicate samples.

CHAPTER 7. METABOLIC REGULATION IN MANDUCA SEXTA

INTRODUCTION

Our current understanding of the changes which occur in carbohydrate and lipid metabolism during the late larval and adult development of Manduca sexta and of the factors which regulate these changes, is principally due to the work of Rolf Ziegler, Karl Siegert and their co-workers. The work reported in this thesis provides additional information on the physiology and pharmacology of the peptide hormone M-AKH which acts as a metabolic regulator in Manduca.

LARVAL METABOLISM AND ITS HORMONAL REGULATION IN MANDUCA

Caterpillar metabolism is primarily anabolic with the ingestion of food permitting growth and the accumulation of energy reserves for subsequent developmental stages (pupa and adult), which depend largely (in the case of the adult) or entirely (in the case of the pupa) on reserves of energy accumulated during the larval stage. However Manduca larvae do not feed continuously (Reinecke et al., 1980) and physiological starvation occurs during the last larval moult (for approximately 24 h) and from the onset of the wandering phase of the fifth and final larval instar until adult eclosion (approximately 25 days later). Additional periods of starvation may be encountered if the food resource becomes scarce and the caterpillar has to actively search for new food (Ziegler, 1985).

During the fifth larval stadium the amount of lipid and carbohydrate stored in the fat body increases from 5 to 180 mg of

triacylglycerol and from 1 to 100 mg of glycogen (Siegert, 1987a). Lipid accumulation is thought to predominate during the first two days of the stadium with glycogen accumulation occurring during days 3 and 4 (Ziegler and Roth, 1985). Larval haemolymph acts as an additional store of carbohydrate as well as a transporting medium, but the haemolymph lipid levels remain low (2 mg/ml) until the wandering phase when they increase to 8-10 mg/ml (Ziegler, 1985). The reason for this increase is not known but it may indicate the release of lipid from the fat body to fuel wandering activity. Trehalose is the major transported carbohydrate in Manduca and its concentration in larval haemolymph is regulated mainly by its synthesis from glucose in the fat body.

The Larval Response to Starvation

The metabolic response to starvation may take the form of an overall reduction in the rate of metabolism and/or the mobilization of stored fuels to permit continued activity. Manduca larvae on day 3 of the fifth instar will reduce their respiration rate by 65-70% within 20 h of the onset of experimental starvation. A similar decrease has been reported during the final larval moult and at the wandering phase (Siegert and Ziegler, 1982). With the exception of the small increase in haemolymph lipid levels at wandering, lipid levels do not change in starved larvae (Ziegler, 1985), but stored carbohydrate is mobilized in response to starvation. The overall change in haemolymph carbohydrate level following the onset of starvation may be quite small and Ziegler (1979) showed that an assay of fat body glycogen phosphorylase activity provided a more

sensitive measure of changes in carbohydrate metabolism. In feeding Manduca larvae around 10% of the fat body phosphorylase is in the active form (phosphorylase a) with the remainder in the inactive form (phosphorylase b). During experimental starvation of day 3 fifth instar larvae the proportion of phosphorylase a increases to 30-50% of the total within 3 h of the onset of starvation. The resultant glucose is converted to trehalose, hence the fall in haemolymph glucose levels even after phosphorylase activation. During developmental starvation glucose liberated by the activation of fat body phosphorylase is used as a substrate for chitin synthesis (Siegert, 1987b).

The activation of phosphorylase is not simply a response to the cessation of feeding activity. Larvae fed on an agar diet showed phosphorylase activation, although feeding movements continued, as they were unable to obtain any nutrient from the diet (Siegert and Ziegler, 1983). The haemolymph trehalose level does not decrease at the onset of starvation and it would therefore seem unlikely that a change in trehalose level acts as the signal for phosphorylase activation. The decrease in haemolymph glucose at the onset of starvation (from 250 µg/ml to less than 100 µg/ml in 3 h) parallels the increase in phosphorylase activity (Siegert, 1987b). Injection of an amount of glucose less than that normally ingested within 3 h, prevented phosphorylase activation in starving larvae whereas injection of trehalose had no such effect. It appeared that a fall in the level of haemolymph glucose may act as a signal for phosphorylase activation. However, phosphorylase activation was not observed when larvae were fed on an agar diet containing 3.2%

sucrose or glucose or fructose or trehalose. In the case of the fructose-fed larvae the haemolymph glucose concentration was similar to that of starved or agar-fed larvae yet the proportion of active phosphorylase remained low. The signal for phosphorylase activation therefore remains unclear (Siegert, 1988).

M-AKH and Larval Starvation

The corpora cardiaca of adult and larval Manduca contain a factor which causes phosphorylase activation when injected into day 3 fifth instar larvae (Ziegler, 1979; Ziegler et al., 1988), but has no effect on larval haemolymph lipid levels (Ziegler, 1984). When the CC were removed from such larvae fat body phosphorylase was not activated in response to experimental starvation. It was concluded that the larval CC contain a factor, referred to as the glycogen phosphorylase activating hormone (GPAH), which regulates larval carbohydrate metabolism in response to starvation (Siegert and Ziegler, 1983). GPAH is produced by the intrinsic neurosecretory cells of the larval CC (Ziegler et al., 1988). Preliminary HPLC analysis suggested that the larval GPAH may be different to the adipokinetic hormone (M-AKH) from adult CC (Ziegler et al., 1986), however recent evidence from amino acid analysis and molecular weight determination suggested that GPAH was identical to M-AKH (Ziegler et al., 1987). My work has shown that larval Manduca CC on day 3 of the fifth instar contain 2 pmol of M-AKH (as defined by radioimmunoassay) and this figure has been used to compare the activity of larval CC extracts reported by Ziegler et al. (1988) with the data reported here for phosphorylase

activation by synthetic M-AKH. The correspondence of the data indicates the likely identity of GPAH with the nonapeptide M-AKH. Ziegler et al. (1988) reported that the GPAH was located exclusively within the CC. Although I found a small amount of M-AKH immunoreactive material in the larval brain and nerve cord this material did not co-elute with M-AKH when analysed by HPLC. It seems unlikely therefore that the material in the brain and nerve cord is M-AKH/GPAH, whereas the CC material was confirmed as M-AKH/GPAH by its co-elution with synthetic M-AKH.

The titre of M-AKH immunoreactive material in the larval haemolymph has been shown to be elevated within 1 h of the onset of experimental starvation (see Chapter 3). Assuming that the majority of this material is M-AKH I have obtained the first direct evidence for the release of M-AKH/GPAH in response to larval starvation. The level of active phosphorylase has been shown to return to the resting level within 24-48 h of starvation despite the continued absence of food (Siegert, 1987b). Phosphorylase inactivation may be due to a reduction in the secretion of GPAH, combined with the inactivation of the hormone, probably by the action of proteolytic enzymes or due to a change in the responsiveness of the fat body despite the continued presence of GPAH. Siegert (1988) injected 24 h and 48 h-starved larvae with CC extract and observed the persistent responsiveness of the fat body. My results also support the former explanation for phosphorylase inactivation as the M-AKH titre declined to the resting level within 3 h of the onset of starvation. It is not known whether a reduction in secretion or

increased degradation is more important in reducing the M-AKH/GPAH titre. A neutral metalloendopeptidase capable of degrading M-AKH has been partially purified from larval haemolymph (see Chapter 6). The *in vivo* function of this enzyme is not known but it seems unlikely that it has a primary role in M-AKH/GPAH inactivation.

Ziegler (1985) has explained the transient nature of the larval response to starvation in terms of a trade-off between growth and development to a subsequent stage. *Manduca* larvae may attain a weight of 10 g by the end of the fifth stadium, however they can pupate successfully and emerge as small adults once they have reached a weight of 4 g (Nijhout, 1975). Consequently a larva weighing 4 g or more when deprived of food will begin to search for food whilst mobilizing stored carbohydrate to fuel this process. If food is not found within a day it will be more energetically efficient for the larva to become quiescent and then pupate to become a moth of reduced size rather than to continue the search for food in an attempt to attain the maximum weight. Hence the inactivation of fat body phosphorylase despite the continuation of starvation. By contrast larvae that are starved early on in the fifth stadium (when they are less than 4 g in weight), will continue to search for food until it is found (they may then moult to a supernumerary larval instar) or until they die (such larvae are too small to pupate successfully).

ADULT METABOLISM AND ITS HORMONAL REGULATION IN *MANDUCA*

The metabolism of the adult moth is primarily catabolic although some synthesis of fuel stores may occur following feeding.

Adult development is not significantly affected by starvation following eclosion and female moths are autogenous, that is they can reproduce despite starvation as adults. The number of eggs laid by a starved female will be greatly reduced compared with the number laid by a fed female (100-200 cf. approximately 1,000 eggs).

The Adult Response to Starvation

In contrast with the larval response, adult fat body glycogen phosphorylase is activated over a period of days rather than hours in response to starvation and there is no evidence of phosphorylase inactivation as starvation continues. Haemolymph glucose levels are very low in adult Manduca and show no correlation with the nutritional state of the moth or the proportion of active phosphorylase. There is a negative correlation between total haemolymph carbohydrate content and the level of active phosphorylase. Since trehalose is the major haemolymph carbohydrate a decrease in haemolymph trehalose may signal the need for phosphorylase activation during adult starvation (Ziegler, 1985). Although CC extract can activate adult fat body phosphorylase (Ziegler, 1984), cardiacectomy does not prevent phosphorylase activation during adult starvation. It would appear that M-AKH/GPAH does not control adult carbohydrate metabolism during starvation. Similarly the increased level of haemolymph lipids in starved moths is not due to the action of M-AKH/GPAH (Ziegler, 1985).

Unlike larvae, moths can afford to mobilize all their reserves of energy during starvation. This may explain the difference between the adult and larval response to starvation. In the larva

changes in one or more haemolymph carbohydrate rapidly signals the onset of starvation leading to a quick response, mediated by GPAH, which lasts for approximately 24 h. In the moth, phosphorylase activation seems to depend upon a decrease in the total level of energy substrate. The adult response is slow (several days) and will last for as long as starvation continues.

Flight Metabolism in Manduca

Carbohydrate metabolism appears to be important during pre-flight warm-up, particularly at low ambient temperatures (Joos, 1987) and at the initiation of flight by Manduca (Ziegler and Schulz, 1986b). During pre-flight warm-up the circulation between the abdomen and the thorax may be restricted so thoracic fuel stores must be utilized. Flight muscle glycogen is degraded during the initial phases of pre-flight warm-up to provide the three- and four-carbon compounds which are required to prime the TCA cycle (Sacktor, 1975).

A sharp decline in the level of haemolymph trehalose has been reported during the first 5 min of tethered flight (Ziegler and Schulz, 1986b). After 5 min the haemolymph trehalose level stabilized due to a reduced rate of utilization (11% of the initial rate). Active fat body glycogen phosphorylase cannot supply the sugar required during early flight at more than 25% of the rate at which it is utilized. It was concluded that the haemolymph pool is the source of most of the carbohydrate used during early flight. The fat body supplies carbohydrate at a reduced rate during sustained flight. Oxygen consumption is very high during the first

2 min of flight (Heinrich, 1971) and carbohydrate thus provides additional energy when it is most needed.

The CC do not regulate carbohydrate metabolism during flight. As with starved moths, phosphorylase activation appears to depend upon a fall in the haemolymph trehalose level, which occurs during the first 5 min of flight (Ziegler and Schulz, 1986b). M-AKH does not therefore appear to be important for the regulation of adult carbohydrate metabolism during starvation or flight.

A comparison of oxygen consumption during flight with changes in haemolymph lipid levels suggests that lipid is the primary fuel for flight in Manduca (Heinrich, 1971; Ziegler and Schulz, 1986a). Haemolymph lipid levels decrease as lipid is utilized during the early stages of flight. After 30 min the rate of lipid mobilization from the fat body equals the rate of utilization by the flight muscles and the level of haemolymph lipid stabilizes (Ziegler and Schulz, 1986a). Even at the minimal value the haemolymph lipid level in adult Manduca is equivalent to the maximum level reported in locusts during flight (Goldsworthy and Cheeseman, 1978).

Cardiacectomized moths rarely fly for longer than 30 min and they do not display the changes in haemolymph lipid levels described above. Ziegler and Schulz (1986a) concluded that lipid mobilization during flight in Manduca was controlled by the nonapeptide hormone, M-AKH from the CC. Radioimmunoassay and HPLC analysis suggest that M-AKH is located exclusively with the CC (approximately 20 pmol/CC, Chapter 3). As with the larval tissue

extracts, RIA analysis of HPLC isolated fractions suggests that the immunoreactive material observed in the adult brain is not M-AKH. The identity of the brain M-AKH-immunoreactive material is not known.

I have compared the adipokinetic activity of synthetic M-AKH in resting moths with that of a CC extract (see Chapter 4). Both agonists produced the same maximum increase in haemolymph lipids (about 30 mg/ml), however the CC extract appeared to be slightly more potent than synthetic M-AKH at low doses. As I obtained only a partial dose-response curve for the CC extract the significance of the difference between the two curves remains uncertain. The difference may be an artefact due to inaccuracies in the preparation of synthetic M-AKH at low doses. This would appear to be the most economical explanation for the data and it would confirm the role of M-AKH as the only adipokinetic hormone in Manduca CC.

However several insect species have been shown to possess two AKH/RPCH peptides. The corn earworm moth, Heliothis zea possesses a nonapeptide identical to M-AKH (Jaffe et al., 1986) and a second AKH/RCH peptide has recently been sequenced from this species (Jaffe et al., 1988b). The difference between the adipokinetic activity of M-AKH and that of CC extract may be due to an additional adipokinetic factor in the CC. A small amount of M-AKH-immunoreactive material was observed in pooled fractions eluting later than M-AKH from a reversed-phase HPLC column. It is

not known whether this material has any adipokinetic activity in Manduca, but it seems unlikely that it represents a second AKH/RPCH peptide in Manduca.

I have investigated the pharmacology of the adipokinetic response in Manduca using synthetic AKH/RPCH family peptides. Only M-AKH was a full agonist but HTF-II, AKH-I and M-II were partial agonists. The serine residues at positions 6 and 7 in M-AKH appear to be essential for full biological activity. A competition assay in which AKH/RPCH peptides were co-injected in ten-fold excess with M-AKH, suggested that M-AKH and the partial agonists bind to the same population of receptors in adult Manduca fat body.

CHAPTER 8. THE POTENTIAL COMMERCIAL APPLICATION OF ADIPOKINETIC HORMONES

THE DEVELOPMENT OF NOVEL INSECTICIDES

The application of insect hormones as novel insecticides was first proposed some thirty years ago, long before the identification of the adipokinetic hormone family (Williams, 1956). At that time studies on the hormones controlling insect metamorphosis had begun to suggest that juvenile hormone (JH) might be developed as an insect-specific pesticide. With the growing concern that insecticides should be arthropod-specific with minimal environmental impact, interest in the development of JH-based insecticides and other methods of biological control increased. The discovery of insect adipokinetic hormones provides another potential source of insect-specific pesticides.

As with the JH-based insecticides, the effect of an AKH-based insecticide would probably be less rapid than conventional neurotoxic insecticides in disabling the target insect. The disruption of flight metabolism by an AKH-based insecticide could be a useful means of controlling locust swarms. A treated locust may not be able to sustain flight for as long as a normal locust and flight speed has been shown to be greatly reduced following CC removal (Goldsworthy and Coupland, 1974).

Manduca is a relatively minor agricultural pest in the United States but Heliothis, which possesses an identical adipokinetic hormone is a major pest of several crops.For lepidopteran pests an AKH-based insecticide might be targeted against the larval stage so

as to disrupt the regulation of carbohydrate metabolism, particularly at the larval-larval moult, when there is a massive flux of carbohydrate through the insect as a result of the digestion of chitin from the old cuticle and the release of carbohydrate from the fat body to act as a substrate for the synthesis of the new cuticle (Siegert, 1987b). The metabolic disturbance caused by an AKH-based insecticide would ideally prevent the larva from moulting successfully and eventually kill it, thus reducing significantly the damage to the host plant caused by the later larval instars.

Unfortunately there are no reports to date on the effects of excessively high or low levels of AKH on insect survival. However a number of studies have investigated the effects of conventional insecticides on AKH activity. A variety of insecticides were shown to cause the abnormally high release of AKH from the locust CC at the paralytic stage of poisoning. It was suggested that the metabolic disturbance and behavioural changes produced by the abnormal release of AKH may contribute to the lethal effect of these insecticides (Samaranayaka, 1974). This study did not indicate whether the insecticides were acting directly on the CC or indirectly through the hyperstimulation of the central nervous system (CNS). More recently a number of conventional insecticides (organochlorines, organophosphates and pyrethroids) have been shown to act directly on the locust CC causing the release of AKH prior to any other poisoning symptoms (Singh and Orchard, 1982, 1983). The CC may be more sensitive to insecticide treatment than the CNS because they are not surrounded by the perineurium but are directly

exposed to any insecticide in the haemolymph (Singh and Orchard, 1982).

It would be naive to imagine that a solution of an AKH peptide could simply be sprayed over a field containing the target insect in order to control it successfully. Peptides are not stable in an agricultural environment and the problem of penetration into the insect is considerable. Quistad et al. (1984) reported insignificant penetration of the cuticle of Manduca larvae by topically applied tritiated proctolin. When Manduca larvae were fed on artificial diet containing tritiated proctolin only 5% of the intact peptide was recovered after 2-5 h whilst substantial quantities of tritiated metabolites were identified. Studies on the locust and the cockroach, as well as the data for Manduca reported here (Chapter 6) suggest that AKH/RPCH family peptides are also susceptible to enzymatic degradation.

A number of alternative strategies for disrupting AKH-regulated metabolism may be suggested (Keeley and Hayes, 1987). The problems of penetration may be overcome by the development of non-peptidic analogues which may act as AKH agonists or antagonists while resisting enzymatic degradation. Structure-activity studies such as those described in Chapter 5 will be of assistance in the development of suitable analogues. Alternatively agents which mimic or antagonise the effects of AKH-releasing agents may be developed. The formamidine insecticide chlordimeform, which is an octopamine agonist, stimulates AKH release in locusts, presumably by binding to octopamine receptors on the CC (Singh et al., 1981). Another possible target would be the system responsible for AKH inactivation. The

haemolymph peptidase described in Chapter 6 may be a part of this system. The disruption of enzyme activity could be a means whereby hormone levels are either artificially increased or reduced.

Perhaps the most interesting possibility is the use of genetic manipulation in the development on an AKH-based insecticide. Once the gene for an AKH/RPCH peptide is identified and sequenced it could be inserted into an insect-specific virus. A solution containing the transformed virus could then be sprayed over the host plant. As the target insect feeds on the host plant it should ingest the virus which can then infect the insect's cells. It has been shown that insect cells infected with a virus containing the gene for human alpha-interferon can express the gene and release interferon into the haemolymph (Maeda et al., 1985). Perhaps this method offers the best possibility for delivering effective quantities of an AKH-based insecticide to the target insect.

Realistically there must be some doubt as to whether the disruption caused by an AKH insecticide would be sufficiently debilitating to make it a commercially viable insecticide. I have therefore indulged in some rather fanciful 'research' into other applications for adipokinetic hormones.

FURTHER APPLICATIONS FOR ADIPOKINETIC HORMONES†

The existence of eccentric entrepreneurs peddling a variety of dubious medicinal treatments has been a feature of many films based in the 'Wild West' of America during the nineteenth century. I was therefore intrigued to discover probably the earliest reference to the pharmaceutical application of adipokinetic hormones in a copy

† The information in this section is entirely fictitious.

of the 'Tucson Post' which I obtained recently from an anonymous source (Fig. 8.1). It is not known whether this preparation had the desired effect on Prof. Ziegler's 'patients'.

More recently a German pharmaceutical company has been marketing a similar product aimed at the health-conscious, marathon-running citizen of the 1980s (Fig. 8.2). I have not been able to contact the manufacturers in order to question them about the precise composition of this product.

Finally, I recently discovered a company of heating engineers who have adopted the name of the adipokinetic hormone family, no doubt because of their interest in improving the energy supply to their clients (Fig. 8.3.). This just goes to show that you truly cannot predict the manner in which pure scientific research will eventually be applied.!

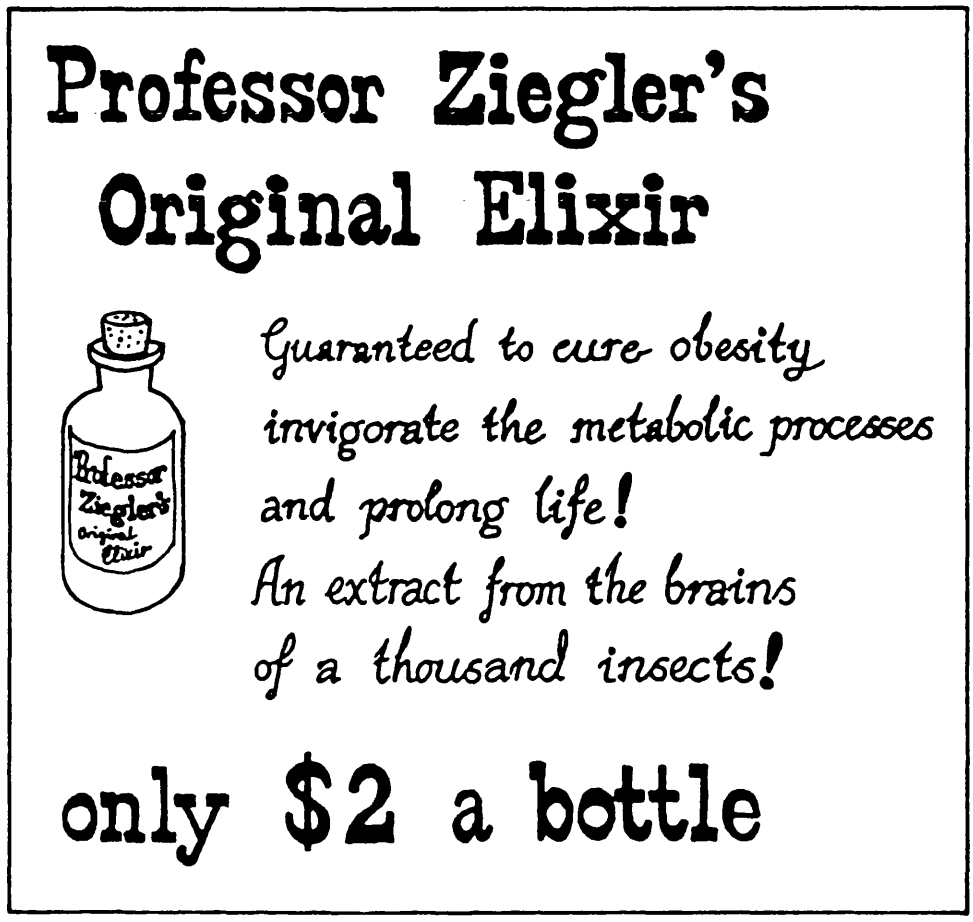


Fig. 8.1. An advertisement from the 'Tucson Post',
25th January 1864.

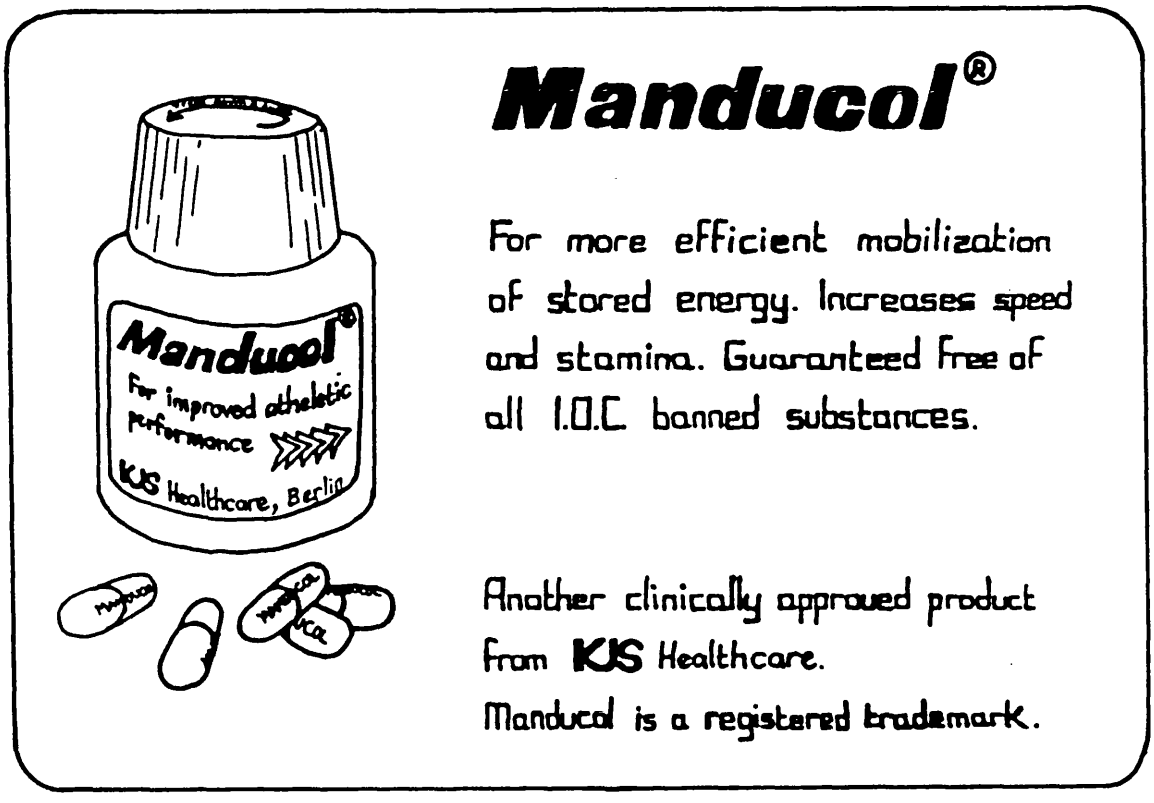


Fig. 8.2. An advertisement from 'Sports Medicine Monthly', September, 1988.

Fig. 8.3. Adipokinetic hormones on the streets of Trowbridge!

REFERENCES

Atherton, E. and Sheppard, R.C. (1987). The fluorenylmethoxy-carbonyl amino protecting group. In: The Peptides: Analysis, Synthesis, Biology. Vol. 9 (Ed. by **Udenfriend S. and Meienhofer, J.**), pp. 1-38. Academic Press, London.

Atherton, E., Caviezel, M., Fox, H., Harkiss, D., Over, H. and Sheppard, R.C. (1983). Peptide synthesis. Part 3. Comparative solid-phase syntheses of human β-endorphin on polyamide supports using t-butoxycarbonyl and fluorenylmethoxycarbonyl protecting groups. J. Chem. Soc. Perkin Trans. I, 65-73.

Barnes, H. and Blackstock, J. (1973). Estimation of lipids in marine animals and tissues: Detailed investigation of the sulphophosphovanillin method for 'total lipids'. J. Exp. Mar. Biol. Ecol. **12**, 103-118.

Barrett, B.A. and Loughton, B.G. (1987). A hypoglycaemic factor from the corpus cardiacum of the American cockroach Periplaneta americana. Gen. Comp. Endocrinol. **66**, 79-84.

Bauer, K. (1985). Degradation and biological inactivation of neuro-peptides. In 'Neurobiochemistry' (Ed. by **Hamprecht B and Neuhoff V.**) pp. 43-54. Springer-Verlag, Berlin.

Baumann, E. and Penzlin, H. (1987). Inactivation of neurohormone D by Malpighian tubules in an insect, Periplaneta americana. J. Comp. Physiol. B **157**, 511-517.

Beenakkers, A.M.T. (1969). The influence of corpus allatum and corpus cardiacum on lipid metabolism in Locusta migratoria. Gen. Comp. Endocrinol. **13**, 492.

Beenakkers, A.M.T. (1973). Influence of flight on lipid metabolism in Locusta migratoria. Insect Biochem. **3,** 303-308.

Beenakkers, A.M.T., Van der Horst, D.J. and Van Marrewijk, W.J.A. (1978). Regulation of release and metabolic function of the adipokinetic hormone in insects. In. Comparative Endocrinology (Ed. by **Gaillard, P.J. and Boer, H.H.**), pp. 445-448. Elsevier/ North Holland, Amsterdam.

Beenakkers, A.M.T., Bloemen, R.E.B., De Vlieger, T.A., Van der Horst, D.J. and Van Marrewijk, W.J.A. (1985a). Insect adipokinetic hormones. Peptides **6 Suppl. 3,** 437-444.

Beenakkers, A.M.T., Van der Horst, D.J. and Van Marrewijk, W.J.A. (1985b). Insect lipids and lipoproteins, and their role in physiological processes. Prog. Lipid Res. **24,** 19-67.

Beenakkers, A.M.T., Van der Horst, D.J. and Van Marrewijk, W.J.A. (1985c). Biochemical processes directed to flight muscle metabolism. In. Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 10 (Ed. by **Kerkut G.A. and Gilbert** L.I.), pp. 451-486. Pergamon Press, Oxford.

Bell, R.A. and Joachim, F.G. (1976). Techniques for rearing laboratory colonies of tobacco hornworm and pink bollworms. Ann. Ent. Soc. Am. **69,** 365-373.

BenKhay, A., Gourdoux, L., Moreau, R. and Dutrieu, J. (1987). Effects of intestinal insulin-like peptide on glucose catabolism in male adult Locusta migratoria. Arch. Insect Biochem. Physiol. **4,** 233-239.

Broomfield, C.E. and Hardy, P.M. (1977). The synthesis of locust adipokinetic hormone. Tetrahedron Lett. **25,** 2201-2204.

Bursell, E. (1963). Aspects of the metabolism of amino acids in the tsetse fly Glossina (Diptera). J. Insect Physiol. **9,** 439-452.

Carlisle, J. and Loughton, B.G. (1979). Adipokinetic hormone inhibits protein synthesis in Locusta. Nature **282,** 420-421.

Carlisle, J. and Loughton, B.G. (1986). The inhibition of protein synthesis in Locusta migratoria by adipokinetic hormone. J. Insect Physiol. **32,** 573-578.

Carlsen, J., Herman, W.S., Christensen, M. and Josefsson, L. (1979). Characterization of a second peptide with adipokinetic and red-pigment concentrating activity from the locust corpora cardiaca. Insect Biochem. **9,** 497-501.

Cheeseman, P., Jutsum, A.R. and Goldsworthy, G.J. (1976). Quantitative studies on the release of locust adipokinetic hormone. Physiol. Entomol. **1,** 115-121.

Downer, R.G.H. (1985). Lipid Metabolism. In. Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 10 (Ed. by **Kerkut G.A. and Gilbert L.I**), pp. 77-113. Pergamon Press, Oxford.

Dryland, A. and Sheppard, R.C. (1986). Peptide synthesis. Part 8. A system for solid-phase synthesis under low pressure continuous flow conditions. J. Chem. Soc. Perkin Trans. I, 125-137.

Duve, H., Thorpe, A. and Lazarus, N.R. (1979). Isolation of material displaying insulin-like immunological and biological activity from the brain of the blowfly Calliphora vomitoria. Biochem. J. **184,** 221-227.

Eberle, A.N. (1987). The Melanotropins. Chemistry, Physiology and Mechanisms of Action. Karger, Basel.

Ephrussi, B. and Beadle, G.W. (1936). A technique for transplantation for Drosophila. Am. nat. **70,** 218-223.

Fernlund, P. and Josefsson, L. (1972). Crustacean color-change hormone: Amino acid sequence and chemical synthesis. Science **177,** 173-175.

Friedman, S. (1985). Carbohydrate metabolism. In. Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 10 (Ed. by **Kerkut G.A. and Gilbert, L.I.**), pp. 43-76. Pergamon Press, Oxford.

Gäde, G. (1986). Relative hypertrehalosaemic activities of naturally occurring neuropeptides from the AKH/RPCH family. Z. Naturforsch. **41c,** 315-320.

Gäde, G. and Holwerda, D. (1976). Involvement of adenosine 3':5'-cyclic monophosphate in lipid mobilization in Locusta migratoria. Insect Biochem. **6,** 535-540.

Gäde, G. and Rinehart, K.L. (1986). Amino acid sequence of a hypertrehalosaemic neuropeptide from the corpus cardiacum of the cockroach, Nauphoeta cinerea. Biochem. Biophys. Res. Comm. **141,** 774-781.

Gäde, G. and Rinehart, K.L. (1987a). Primary structure of the hypertrehalosaemic factor II from the corpus cardiacum of the Indian stick insect, Carausius morosus, determined by fast atom bombardment mass spectrometry. Biol. Chem. Hoppe-Seyler **368,** 67-75.

Gäde, G. and Rinehart, K.L. (1987b). Primary sequence analysis by fast atom bombardment mass spectrometry of a peptide with adipokinetic activity from the corpora cardiaca of the cricket, Gryllus bimaculatus. Biochem. Biophys. Res. Comm. **149,** 908-914.

Gäde, G., Goldsworthy, G.J., Schaffer, M.H., Cook, J.C. and Rinehart, K.L. (1986). Sequence analyses of adipokinetic hormones II from corpora cardiaca of Schistocerca nitans, Schistocerca gregaria and Locusta migratoria by fast atom bombardment mass spectrometry. Biochem. Biophys. Res. Comm. **134,** 723-730.

Gäde, G., Hilbich, C., Beyreuther, K. and Rinehart, K.L. (1988). Sequence analyses of two neuropeptides of the AKH/RPCH-family from the lubber grasshopper, Romalea microptera. Peptides **9,** 681-688.

Goldsworthy, G.J. and Cheeseman, P. (1978). Comparative aspects of the endocrine control of energy metabolism. In. Comparative Endocrinology (Ed. by **Gaillard P.J. and Boer H.H.**), pp. 423-436. Elsevier/North Holland, Amsterdam.

Goldsworthy, G.J. and Coupland, A.J. (1974). The influence of the corpora cardiaca and substrate availability on flight speed and wing beat frequency in Locusta. J. Comp. Physiol. **89,** 359-368.

Goldsworthy, G.J. and Wheeler, C.H. (1986). Structure/Activity relationships in the adipokinetic hormone/red-pigment concentrating hormone family. In Insect Neurochemistry and Neurophysiology 1986 (Ed. by **Borkovec A.B. and Gelman, D.B.**), pp. 183-186, Humana Press, New Jersey.

Goldsworthy, G.J., Mordue, W. and Guthkelch, J. (1972). Studies on insect adipokinetic hormones. Gen. Comp. Endocrinol. **18,** 545-551.

Goldsworthy, G.J., Mallison, K. and Wheeler, C.H. (1986a). The relative potencies of two known locust adipokinetic hormones. J. Insect Physiol. **32,** 95-101.

Goldsworthy, G.J., Mallison, K., Wheeler, C.H. and Gäde, G. (1986b). Relative adipokinetic activities of members of the adipokinetic/red pigment concentrating hormone family. J. Insect Physiol. **32,** 433-438.

Gourdoux, L., Moreau, R., Ben Khay, A. and Dutrieu, J. (1985). In vivo glucose catabolism in male adult Locusta migratoria. Effects of corpora cardiaca. Comp. Biochem. Physiol. **81B,** 485-489.

Hardy, P.M. and Sheppard, P.W. (1983). Synthesis of [4,5-dehydroLeu^{2}]- and [3,4-dehydroPro^{6}]-locust adipokinetic hormone. J. Chem. Soc. Perkin Trans. I, 723-729.

Hayes, T.K., Keeley, L.L. and Knight, D.W. (1986). Insect hypertrehalosaemic hormone: Isolation and primary structure from Blaberus discoidalis cockroaches. Biochem. Biophys. Res. Comm. **140,** 674-678.

Heinrich, B. (1971). Temperature regulation of the sphinx moth, Manduca sexta. I. Flight energetics and body temperature during free and tethered flight. J. Exp. Biol. **54,** 141-152.

Hekimi, S. and O'Shea, M. (1987). Identification and purification of two precursors of the insect neuropeptide adipokinetic hormone. J. Neurosci **7,** 2773-2784.

Holman, G.M., Cook, B.J. and Nachman, R.J. (1986). Isolation, primary structure and synthesis of two neuropeptides from Leucophaea maderae: members of a new family of cephalomyotropins. Comp. Biochem. Physiol. **84C**, 205-211.

Isaac, R.E. (1987). Proctolin degradation by membrane peptidases from nervous tissues of the desert locust (Schistocerca gregaria). Biochem. J. **245**, 365-370.

Isaac, R.E. (1988). Neuropeptide-degrading endopeptidase activity of locust (Schistocerca gregaria) synaptic membranes. Biochem. J. 255, 843-847.

Jaffe, H., Raina, A.K., Riley, C.T., Fraser, B.A., Holman, G.M., Wagner, R.M., Ridgway, R.L. and Hayes, D.K. (1986). Isolation and primary structure of a peptide from the corpora cardiaca of Heliothis zea with adipokinetic activity. Biochem. Biophys. Res. Comm. **135**, 622-628.

Jaffe, H., Raina, A.K., Fraser, B.A., Keim, P., Rao, K.R., Zhang, Y.-S., Lancaster, J.L. and Hayes, D.K. (1988a). Isolation of two neuropeptides in the AKH/RPCH family from horseflies (Diptera). Biochem. Biophys. Res. Comm. **151**, 656-663.

Jaffe, H., Raina, A.K., Riley, C.T., Fraser, B.A., Bird, T.G., Tseng, C.-M., Zhang, Y.-S. and Hayes, D.K. (1988b). Isolation and primary structure of a neuropeptide from Heliothis zea with hypertrehalosemic and adipokinetic activities. Biochem. Biophys. Res. Comm. **155**, 344-350.

Jongbloed, J. and Wiersma, C.A.G. (1934). Der Stoffwechsel der Honig-biene wahrend des Fluges. Z. Vergl. Physiol. **21**, 519-533.

Joos, B. (1987). Carbohydrate use in the flight muscles of Manduca sexta during pre-flight warm-up. J. Exp. Biol. **133,** 317-327.

Kaiser, E., Colescott, R.L., Bossinger, C.D. and Cook, P.I. (1970). Color test for detection of free terminal amino-groups in the solid-phase synthesis of peptides. Anal. Biochem. **34,** 595-598.

Keeley, L.L. and Hayes, T.K. (1987). Speculations on biotechnology applications for insect neuroendocrine research. Insect Biochem. **17,** 639-651.

Kenny, A.J. (1986). Cell surface peptidases are neither peptide- nor organ-specific. Trends Biochem. Sci. **11,** 40-42.

Kisfaludy, L., Korenczki, F., Mohacsi, T., Sajgo, M. and Fermandjian, S. (1986). A new solution synthesis of locust adipokinetic hormone. Int. J. Peptide Protein Res.. **27,** 440-442.

Kramer, K.J., Childs, C.N., Spiers, R.D. and Jacobs, R.M. (1982). Purification of insulin-like peptides from insect haemolymph and royal jelly. Insect Biochem. **12,** 91-98.

Kramer, K.J. (1985). Vertebrate hormones in insects. In. Comprehensive Insect Physiology Biochemistry and Pharmacology, Vol. 7 (Ed. by **Kerkut G.A. and Gilbert, L.I.**), pp. 511-536. Pergamon Press, Oxford.

Krogh, A. and Weis-Fogh, T. (1951). The respiratory exchange of the desert locust (Schistocerca gregaria) before, during and after flight. J. Exp. Biol. **28,** 344-357.

Loughton, B.G. (1987). Recovery from hyperlipaemia in the locust. J. Insect Physiol. **33,** 663-667.

Loughton, B.G. (1988). The mode of action of locust hypolipaemic hormone. Pestic. Sci., **24**, 260-263.

Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, Y., Sato, Y. and Furusawa, M. (1985). Production of human alpha-interferon in silkworm using a baculovirus vector. Nature **351**, 592-594.

Mayer, R.J. and Candy, D.J. (1969). Control of haemolymph lipid concentration during locust flight: an adipokinetic hormone from the corpora cardiaca. J. Insect Physiol. **15**, 611-620.

McKelvey, J.F. and Blumberg, S. (1986). Inactivation and metabolism of neuropeptides. Annu. Rev. Neurosi., **9**, 415-434.

Mead, G.P., Ratcliffe, N.A. and Renwrantz, L.R. (1986). The separation of insect haemocyte types on percoll gradients: methodology and problems. J. Insect Physiol. **32**, 167-177.

Merrifield, B. (1986). Solid phase synthesis. Science **232**, 341-347.

Mordue, W. and Stone, J.V. (1978). Structure and metabolism of adipokinetic hormones. In: Comparative Endocrinology (Ed. by **Gaillard, P.J. and Boer, H.H.**), pp. 487-490. Elsevier/North Holland, Amsterdam.

Moshitzky, P., Yamashiro, D., Stuve, L., Ramachandran, J. and Applebaum, S.W. (1987a). Determination of locust AKH-I by radioimmunoassay and the identification of an AKH-like factor in the locust brain. Insect Biochem. **17**, 765-769.

Moshitzky, P., Henzel, W.J., Rafaeli, A., Ramachandran, J. and Applebaum, S.W. (1987b). Synthesis of adipokinetic hormone (AKH-I) in the locust brain. Insect Biochem. **17**, 1133-1137.

Muramoto, K., Ramachandran, J., Moshitzky, P. and Applebaum, S.W. (1984). Preparation of a specifically tritiated locust adipokinetic hormone analog with full biological potency. Int. J. Peptide Protein Res. **23**, 443-446.

Nijhout, H.J.F. (1975). A threshold size for metamorphosis in the tobacco hormworm, Manduca sexta (L.). Biol. Bull. (Woods Hole) **149**, 214-225.

Orchard, I. (1987). Adipokinetic hormones - an update. J. Insect Physiol., **33**, 451-463.

Orchard, I. and Loughton, B.G. (1980). A hypolipaemic factor from the corpous cardiacum of locusts. Nature **286**, 494-496.

Orchard, I. and Loughton, B.G. (1981). The neural control of release of hyperlipaemic hormone from the corpus cardiacum of Locusta migratoria. Comp. Biochem. Physiol. **68A**, 25-30.

Orlowski, M., Michaud, C. and Chu, T.G. (1983). A soluble metallo-endopeptidase from rat brain. Purification of the enzyme and determination of specificity with synthetic and natural peptides. Eur. J. Biochem., **135**, 81-88.

O'Shea, M. and Adams, M. (1986). Proctolin: from "gut factor" to model neuropeptide.Adv. Insect Physiol. **19**, 1-28.

O'Shea, M., Witten, J.L. and Schaffer, M. (1984). Isolation and characterization of two myoactive neuropeptides: Further evidence of an invertebrate peptide family. J. Neurosci. **4**, 521-529.

Pannabecker, T. and Orchard, I. (1986). Octopamine and cyclic AMP mediate release of adipokinetic hormone I and II from isolated neuroendocrine tissue. Mol. Cell. Endocrinol. **48**, 153-158.

Platt, N. and Reynolds, S.E. (1985). Cardioactive peptides from the CNS of a caterpillar, the tobacco hornworm, Manduca sexta. J. Exp. Biol. **114**, 397-414.

Platt, N. and Reynolds, S.E. (1986). The pharmacology of the heart of a caterpillar, the tobacco hornworm, Manduca sexta. J. Insect. Physiol. **32**, 221-230.

Prosser, H.M. (1987). Protein synthesis by the fat body of Manduca sexta: an investigation of a possible regulatory role for adipokinetic hormone. Project report for the Honours degree in Applied Biology, University of Bath.

Quistad, G.B., Adams, M.E., Scarborough, R.M., Carney, R.L. and Schooley, D.A. (1984). Metabolism of proctolin, a pentapeptide neurotransmitter in insects. Life Sci., **34**, 569-576.

Reinecke, P., Buckner, J.S. and Grugel, S.R. (1980). Life cycle of laboratory-reared tobacco hornworms, Manduca sexta, a study of development and behaviour, using time lapse cinematography. Biol. Bull. (Woods Hole) **158**, 129-140.

Robinson, N.L. and Goldsworthy, G.J. (1977). A possible site of action for adipokinetic hormone on the flight muscle of locusts. J. Insect Physiol. **23**, 153-158.

Rowan, A.N. and Newsholme, E.A. (1979). Changes in the contents of adenine nucleotides and intermediates of glycolysis and the citric acid cycle in flight muscle of the locust upon flight and their relationship to control of the cycle. Biochem. J. **178**, 209-216.

Sacktor, B. (1975). Biochemistry of insect flight. In Insect Biochemistry and Function. (Ed. by **Candy, D.J. and Kilby,**

B.A.), pp. 1-88, Wiley, New York.

Samaranayaka, M. (1974). Insecticide-induced release of hyperglycaemic and adipokinetic hormones of Schistocerca gregaria. Gen. Comp. Endocrinol. **24,** 424-436.

Sarin, V.K., Kent, S.B.H., Tam, J.P. and Merrifield, R.B. (1981). Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. Anal. Biochem. **117,** 147-157.

Scarborough, R.M., Jamieson, G.C., Kalish, F., Kramer, S.J., McEnroe, G.A., Miller, C.A. and Schooley, D.A. (1984). Isolation and primary structure of two peptides with cardioacceleratory and hyperglycaemic activity from the corpora cardiaca of Periplaneta americana. Proc. Natl. Acad. Sci. USA. **81,** 5575-5579.

Schooneveld, H., Tesser, G.I., Veenstra, J.A. and Romberg-Privee, H.M. (1983). Adipokinetic hormone and AKH-like peptide demonstrated in the corpora cardiaca and nervous system of Locusta migratoria by immunocytochemistry. Cell Tissue Res. **230,** 67-76.

Schooneveld, H., Romberg-Privee, H.M. and Veenstra, J.A. (1986). Phylogenetic differentiation of glandular cells in corpora cardiaca as studied immunocytochemically with region-specific antisera to adipokinetic hormone. J. Insect Physiol. **33,** 167-176.

Shapiro, J.P., Law, J.H. and Wells, M.A. (1988). Lipid transport in insects. Ann. Rev. Entomol. **33,** 297-318.

Siegert, K.J. (1987a). Carbohydrate metabolism in Manduca sexta during late larval development. J. Insect Physiol. **33,** 421-427.

Siegert, K.J. (1987b). Carbohydrate metabolism in starved fifth instar larvae of Manduca sexta. Arch. Insect Biochem. Physiol. **4**, 151-160.

Siegert, K.J. (1988). Hormonal regulation of fat body glycogen phosphorylase activity in larval Manduca sexta during starvation. Gen. Comp. Endocrinol. **71**, 205-211.

Siegert, K.J. and Ziegler, R. (1982). Sauerstoffverbrauch und respiratorische Quotienten bei Manduca sexta (Lepidoptera: Sphingidae). Verh. Dtsch. Zool. Ges. 1982, 332.

Siegert, K.J. and Ziegler, R. (1983). A hormone from the corpora cardiaca controls fat body glycogen phosphorylase during starvation in tobacco hornworm larvae. Nature **301**, 526-527.

Siegert, K.J. and Mordue, W. (1986). Quantification of adipokinetic hormones I and II in the corpora cardiaca of Schistocerca gregaria and Locusta migratoria. Comp. Biochem. Physiol. **84A**, 279-284.

Siegert, K.J. and Mordue, W. (1987). Breakdown of locust adipokinetic hormone I by Malpighian tubules of Schistocerca gregaria. Insect Biochem. **17**, 705-710.

Siegert, K., Morgan, P. and Mordue, W. (1985). Primary structures of locust adipokinetic hormones II. Biol. Chem. Hoppe-Seyler **366**, 723-727.

Singh, G.J.P. and Orchard, I. (1982). Is insecticide-induced release of insect neurohormones a secondary effect of hyperactivity on the central nervous system? Pestic. Biochem. Physiol. **17**, 232-242.

Singh, G.J.P. and Orchard, I. (1983). Action of bioresmethrin on the corpus cardiacum of Locusta migratoria. Pestic. Sci. **14,** 229-234.

Singh, G.J.P., Orchard, I. and Loughton, B.G. (1981). Octopamine-like action of formamidines on hormone release in the locust, Locusta migratoria. Pestic. Biochem. Physiol. **16,** 249-255.

Skinner, W., Quistad, G.B., Adams, M.E. and Schooley, D.A. (1987). Metabolic degradation of the peptide periplanetin CC-2 in the cockroach, Periplaneta americana. Insect Biochem. **17,** 433-437.

Starratt, A.N. and Steele, R.W. (1984). In vivo inactivation of the insect neuropeptide proctolin in Periplaneta americana. Insect Biochem. **14,** 97-102.

Steele, J.E. (1961). Occurrence of a hyperglycaemic factor in the corpus cardiacum of an insect. Nature **192,** 680-681.

Steele, R.W. and Starratt, A.N. (1985). In vitro inactivation of the insect neuropeptide proctolin in haemolymph from Periplaneta americana. Insect Biochem. **15,** 511-519.

Stone, J.V., Mordue, W., Batley, K.E. and Morris, H.R. (1976). Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. Nature **263,** 207-211.

Stone, J.V., Mordue, W., Broomfield, C.E. and Hardy, P.M. (1978). Structure-activity relationships for the lipid mobilising action of locust adipokinetic hormone. Synthesis and activity of a series of hormone analogues. Eur. J. Biochem. **89,** 195-202.

Tager, H.S., Markese, J., Kramer, K.J., Spiers, R.D. and Childs, C.N. (1976). Glucagon-like and insulin-like hormones of the insect neurosecretory system. Biochem. J. **156,** 515-520.

Turner, A.J., Matsas, R. and Kenny, A.J. (1985). Are there neuropeptide specific peptidases? Biochem. Pharmacol. **34**, 1347-1356.

Van Marrewijk, W.J.A., Van den Broek, A.T.M. and Beenakkers, A.M.T. (1983). Regulation of glycogen phosphorylase activity in the fat body of Locusta migratoria and Periplaneta americana. Gen. Comp. Endocrinol. **50**, 226-234.

Van Marrewijk, W.J.A., Van Den Broek, A.T.M. and Beenakkers, A.M.T. (1986). Hormonal control of fat-body glycogen mobilization for locust flight. Gen. Comp. Endocrinol. **64**,, 136-142.

Wheeler, C.H. and Goldsworthy, G.J. (1985a). Lipid transport to the flight muscles in Locusta. In: Insect Locomotion (Ed. by **Gewecke, M. and Wendler, G.**) pp. 126-135. Paul Parey Press, Berlin.

Wheeler, C.H. and Goldsworthy,G.J. (1985b). Specificity and localisation of lipoprotein lipase in the flight muscles of Locusta migratoria. Biol. Chem. Hoppe-Seyler **366**, 1071-1078.

Wheeler, C.H., Gäde, G. and Goldsworthy, G.J. (1988). Humoral functions of insect neuropeptides. In: Neurohormones in Invertebrates (Ed. by **Thorndyke M.C. and Goldsworthy, G.J.**) pp. 141-157. Cambridge University Press, Cambridge.

Williams, C.M. (1956). The juvenile hormones of insects. Nature **178**, 212-213.

Witten, J.L., Schaffer, M.H., O'Shea, M., Cook, J.C., Hemling, M.E. and Rinehart, K.L. (1984). Structures of two cockroach neuropeptides assigned by fast atom bombardment mass spectrometry. Biochem. Biophys. Res. Comm. **124**, 350-358.

Wyatt, G.R. and Kalf, G.F. (1957). The chemistry of insect haemolymph. II. Trehalose and other carbohydrates. J. Gen. Physiol. **40**, 833-847.

Yamashiro, D., Applebaum, S.W., Birk, Y. and Li, C.H. (1981). Solid phase synthesis of locust adipokinetic hormone. Int. J. Peptide Protein Res. **17**, 546-548.

Zebe, E. (1953). Uber den respiratorischen Quotienten der Lepidopteren. Naturwissenschaften **40**, 298.

Ziegler, R. (1979). Hyperglycaemic factor from the corpora cardiaca of Manduca sexta (L.) (Lepidoptera. Sphingidae). Gen. Comp. Endocrinol. **39**, 350-357.

Ziegler, R. (1984). Developmental changes in the response of the fat body of Manduca sexta to injections of corpora cardiaca extracts. Gen. Comp. Endocrinol. **54**, 51-58.

Ziegler, R. (1985). Metabolic energy expenditure and its hormonal regulation. In: Environmental Physiology and Biochemistry of Insects (Ed. by **Hoffman, K.H.**) pp. 95-118. Springer-Verlag, Berlin.

Ziegler, R. and Gåde, G. (1984). Preliminary characterization of glycogen phosporylase activating hormone and adipokinetic hormone from Manduca sexta corpora cardiaca. Physiol. Entomol. **9**, 229-236.

Ziegler, R. and Roth, K. (1985). ^{13}C-NMR spectroscopy of larvae of Manduca sexta in vivo. Naturwissenschaften **72**, 206-207.

Ziegler, R. and Schulz, M. (1986a). Regulation of lipid metabolism during flight in Manduca sexta. J. Insect Physiol. **32**, 903-908.

Ziegler, R. and Schulz, M. (1986b). Regulation of carbohydrate metabolism during flight in Manduca sexta. J. Insect Physiol. **32**, 997-1001.

Ziegler, R., Ashida, M., Fallon, A.M., Wimer, L.T., Wyatt, S.S. and Wyatt, G.R. (1979). Regulation of glycogen phosphorylase in fat body of Cecropia silk moth pupae. J. Comp. Physiol. **131**, 321-332.

Ziegler, R., Kegel, G. and Keller, R. (1984). Isolation and amino acid composition of the adipokinetic hormone of Manduca sexta. Hoppe-Seyler's Z. Physiol. Chem. **365**, 1451-1456.

Ziegler, R., Eckart, K., Schwarz, H. and Keller, R. (1985). Amino acid sequence of Manduca sexta adipokinetic hormone elucidated by combined fast atom bombardment (FAB)/tandem mass spectrometry. Biochem. Biophys. Res. Comm. **133**, 337-342.

Ziegler, R., Kegel, G. and Keller, R. (1986). AKH and GPAH are not identical in Manduca sexta. J. Cell. Biochem. Suppl. **10C**, 71.

Ziegler, R., Eckart, K., Kingan, T. and Law, J.H. (1987). Adipokinetisches Hormon und glykogenphosphorylase-aktivierendes Hormon in Manduca sexta. Verh. Dtsch. Zool. Ges. 1987, 185-186.

Ziegler, R., Hoff, R. and Rohde, M. (1988). Storage site of glycogen phosphorylase activating hormone in larvae of Manduca sexta. J. Insect Physiol. **34**, 143-150.